THE HUMAN TYPE 5, TARTRATE-RESISTANT ACID PHOSPHATASE: PURIFICATION, CHARACTERIZATION AND MOLECULAR CLONING

By

CATHERINE MARY KETCHAM

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ABBREVIATIONS

bp, base pairs

BSA, bovine serum albumin

cDNA, complementary DNA

CM-cellulose, carboxymethyl cellulose

ConA, Concanavalin A

DME, Dulbecco's Modified Eagle's Medium

DME-HAT, DME with hypoxanthine, aminopterin and thymidine

DNase, deoxyribonuclease

DTT, dithiothreitol

EBV, Epstein-Barr Virus

EDTA, ethylenediaminetetraacetic acid

GlcNAc, N-acetylglucosamine

GTC, guanidinium thiocyanate solution

IgG, immunoglobulin G

i.p., intraperitoneally

IPTG, isopropylthio-β-D-galactopyranoside

kb, kilobases

K_D, dissociation constant

MOPS, 3-(N-morpholino)propanesulfonic acid

Mr, relative molecular mass

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline

Phosphodiesterase, N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase

Phosphotransferase, UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine 1-phosphate transferase

PMSF, phenylmethylsulfonylfluoride

pNPP, p-nitrophenylphosphate

RNase, ribonuclease

SDS, sodium dodecyl sulfate

SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate

SP2/0, SP2/0-Ag14

SSC, standard sodium citrate buffer

TE, Tris-HCl with EDTA

TPA, 12-0-tetradecanoylphorbol 13-acetate

TR-AP, tartrate resistant acid phosphatase (Type 5 isozyme)

Tris, tris(hydroxymethyl)aminomethane

Uf, uteroferrin

X-gal, 5-chloro-4-bromo-3-indolyl β-D-galactopyranoside

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Catherine Mary Ketcham

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Major Department: Biochemistry and Molecular Biology

The spleens of patients with hairy cell leukemia contain high levels of a tartrate-resistant cationic acid phosphatase, known as the Type 5 isozyme. This enzyme is also present in small quantities in normal spleen and term placenta. This phosphatase has been purified from these three sources by a procedure which involves only two chromatographic steps: CM-cellulose chromatography and immunoaffinity chromatography on a column of anti-uteroferrin antibodies. Uteroferrin is an abundant purple, iron-containing acid phosphatase that can be easily purified from porcine uterine secretions. Like uteroferrin, the human enzyme is an iron-containing glycoprotein of apparent molecular weight 34,000. The human phosphatase and uteroferrin also resemble each other closely in electrophoretic mobility, substrate specificity, and response to a variety of activators and inhibitors. Three anti-uteroferrin monoclonal antibodies which bind with high affinities to distinct sites on the uteroferrin molecule also recognize the human spleen enzyme, but bind to

it with much lower affinities. These antibodies also recognize purple, iron-containing acid phosphatases from bovine and rat spleens and the bovine uterus. A 1412-base pair cDNA has been cloned from human placenta which encodes the entire human tartrate-resistant, Type 5 isozyme. This cDNA contains an open reading frame of 969 base pairs, corresponding to a protein of 323 amino acids. A putative signal sequence of 19 amino acids and two potential glycosylation sites are present. The deduced amino acid sequence of the human enzyme is 85% identical to that of porcine uteroferrin and 82% identical to the corresponding regions of a partial amino acid sequence of the bovine spleen enzyme. Northern blotting techniques employing a radiolabeled cDNA probe coding for the human enzyme revealed the presence of a 1.5 kb transcript in leukemic hairy cells, an Epstein-Barr virus-transformed B-cell line, and the erythroleukemia cell line K562. Culture of K562 cells in the presence of 10^{-8}M 12-0-tetradecanoylphorbol 13-acetate enhanced tartrate-resistant acid phosphatase activity about 30-fold and led to a corresponding increase in Type 5 isozyme mRNA levels.

CHAPTER 1
INTRODUCTION

The Tartrate-resistant Acid Phosphatases

The tartrate-resistant acid phosphatases have been studied extensively by various research groups with different objectives. The most abundant enzyme in this family, porcine uteroferrin, has been well characterized physically and enzymatically, yet much of the research on this protein has involved the determination of its role in iron metabolism in the mid-pregnant pig. The beef spleen acid phosphatase was first identified over 25 years ago, and while its physical characteristics as a metalloprotein are well known, its function remains obscure. The human tartrate-resistant acid phosphatase was first described by researchers interested in the clinical significance of phosphatase levels in normal and pathological tissue. Few reports have been published on the characterization of this human enzyme. It is clear, however, that all of the above proteins share several unusual features, and that the clinically relevant human enzyme is worthy of further study.

Uteroferrin

The uterine secretions of pigs contain a purple, basic, iron-containing glycoprotein with acid phosphatase activity known as uteroferrin. Uteroferrin has been well characterized since it was first described in 1972 by Murray et al. Its enzymatic characteristics have

been studied by our laboratory, and its interesting spectral properties have been the subject of numerous publications by many different researchers. Our laboratory has also determined the likely function of uteroferrin in the pregnant pig.

Uteroferrin is Synthesized by the Uterine Endometrium Under the Influence of Progesterone

One of the major goals of our laboratory has been to purify and characterize the proteins secreted by the porcine uterus. The quantity and variety of proteins produced depend on the hormonal state of the animal. Knight et al. (1974) described the collection of uterine fluids from ovariectomized gilts which had been treated with estradiol 17-β, progesterone, progesterone plus estradiol 17-β, or corn oil (control), for 15 days. It was demonstrated that progesterone or progesterone plus estradiol treatment caused a highly significant increase in the amount of total protein that could be recovered relative to corn oil control or estradiol treatment alone. Estradiol alone promoted neither increased protein accumulation nor the appearance of novel proteins, but it acted synergistically with progesterone to stimulate uterine secretory activity when given at low dosage. A variety of proteins were thus shown to be secreted by the porcine uterus under the influence of progesterone. A protein now known as uteroferrin accounted for about 10-15% of the total protein in the uterine secretions of progesterone treated animals (Schlosnagle et al., 1974).

Simmen et al. (1988) compared the biosynthetic rate of uteroferrin to uteroferrin mRNA levels throughout pregnancy in the pig. Synthesis of uteroferrin begins to increase markedly after day 30 of pregnancy in association with a decline in maternal estrogen levels. Uteroferrin

biosynthesis reaches a maximum between days 60 and 75 (Basha et al., 1979), and then declines towards term, which is about day 115 (Bazer et al., 1975). The progesterone to estrogen ratios are highest between days 35 to 75, which coincides well with maximal uteroferrin production (Roberts and Bazer, 1980). Levels of uteroferrin in allantoic fluid during pregnancy reach a maximum at a similar time (Roberts and Bazer, 1980). In comparison, highest levels of mRNA coding for uteroferrin were detected during mid- and late-pregnancy. The mRNA levels at days 45-60 were comparable to those at days 90-110 (Simmen et al., 1988). Thus, the amount of uteroferrin mRNA as determined by Northern analysis does not appear to reflect the rate of uteroferrin biosynthesis as determined by the incorporation of radioactive leucine. The regulation of uteroferrin synthesis may therefore involve transcriptional and post-transcriptional control. These results support the earlier theory that although progesterone is essential for uteroferrin production, estrogens may have an important modulating role, due to the fact that uteroferrin synthesis is highest when the ratio of progesterone to estrogen is highest (Roberts and Bazer, 1980).

The Purification of Uteroferrin from Uterine Secretions

Uteroferrin is most conveniently purified from the uterine flushes of pseudopregnant animals (Basha et al., 1980), or from the allantoic fluid of mid-pregnant pigs (Bazer et al., 1975). Pseudopregnancy can be induced in sows by administering estradiol-17β daily between days 11-14 after estrus (Frank et al., 1978). The injected estradiol appears to mimic an estrogen burst from the blastocyst which is believed to be the signal to the sow which indicates she is pregnant (Bazer and Thatcher,

1977). Secretions of a day 60 pseudopregnant animal are comparable in quality and quantity to those of an ovariectomized animal given daily doses of progesterone for two months. Uteroferrin can be purified from allantoic fluid after day 30 of pregnancy. However, uteroferrin in sterile allantoic fluid is rapidly degraded, even when stored at 4°C. Therefore, the allantoic fluid must be frozen after collection or used immediately, and the purification of uteroferrin must be carried out quickly.

Because uteroferrin is available in gram quantities, and because of its purple color, the development of a purification protocol for uteroferrin was quite straightforward. Since uteroferrin is a very basic protein, it binds to carboxymethyl cellulose even at high pH values. A combination of ion exchange chromatography and gel filtration on Sephadex G-100 is sufficient to yield pure uteroferrin of Mr 35,000, as determined by SDS polyacrylamide gel electrophoresis (Chen et al., 1973). Interestingly, uterine flushes and allantoic fluid obtained from animals during early pregnancy (days 45-60) also contain a high molecular weight (Mr=80,000), pink form of uteroferrin (Bazer et al., 1975). It is now known that this protein consists of a molecule of uteroferrin non-covalently associated with another, antigenically unrelated protein (Baumbach et al., 1986). The reason for the association of uteroferrin with this second protein is unknown.

A number of other interesting proteins, though none as abundant as uteroferrin, can also be purified from porcine uterine secretions (see Roberts and Bazer, 1988). These proteins include a family of trypsin/plasmin protease inhibitors (Mullins et al., 1980; Fazleabas et

al., 1982), and a group of retinol binding proteins (Adams et al., 1981).

Uteroferrin is an Iron-containing Acid Phosphatase

Schlosnagle et al. (1974) demonstrated that the abundant, basic, purple glycoprotein from porcine uterine secretions was an iron-containing acid phosphatase. They reported a pH optimum of 4.9 and a Km of 2.2mM for the enzyme's preferred substrate, p-nitrophenylphosphate. Weak reducing agents, such as 2-mercaptoethanol enhanced activity 2- to 4-fold, without changing the Km. Uteroferrin's phosphatase activity could be inhibited by fluoride, arsenate, phosphate and molybdate (Schlosnagle et al., 1976). Sodium dithionite caused an immediate loss of enzymatic activity and bleaching of color, although activity could be restored with Fe^{3+} salts (Schlosnagle et al., 1976). Oxidizing agents, and reagents which interact with sulfhydryl groups also inhibited uteroferrin (Schlosnagle et al., 1976). Uf was not inhibited by tartrate, an unusual feature in an acid phosphatase. The substrate specificity of uteroferrin was also unusual. It readily hydrolyzed compounds with strong leaving groups, such as p-nitrophenylphosphate and ATP, but had little or no activity towards aliphatic phosphates such as β-glycerol phosphate, or hexose phosphates such as D-glucose 6-phosphate (Schlosnagle et al., 1974). Uteroferrin also displayed phosphoprotein phosphatase activity (Roberts and Bazer, 1976).

Uteroferrin, as purified, is predominantly purple and enzymatically inactive. This purple coloration originates from a broad absorption band centered around 545nm. However, when uteroferrin is activated by 2-mercaptoethanol, the extinction maximum changes from 545nm (purple) to

508nm (pink) (Schlosnagle et al., 1974). The reverse reaction (pink to purple) is promoted by oxidizing agents. When purified, uteroferrin consists of an equilibrium mixture of the reduced (pink) and oxidized (purple) forms, with the latter predominating. The equilibrium between the pink and purple forms may depend on the redox state of the fluid sample from which the protein was recovered. In contrast, the stable pink, high molecular weight form of uteroferrin does not require reducing agents for activity, it is fully active as purified (Baumbach et al., 1986). It is not clear how non-covalent association with a second, unrelated protein stabilizes the active form of the enzyme.

Uteroferrin Contains Two Iron-binding Sites

The amount of iron bound to uteroferrin has been the subject of controversy. Early reports (Roberts and Bazer, 1980) indicated that uteroferrin contained a monoferric site. However, it is now clear that each molecule of uteroferrin can bind up to two iron atoms, although only one of these iron atoms is necessary for the purple/pink coloration. It has been demonstrated that the iron at the more labile, non-chromophoric site can be replaced by a variety of other metals including zinc, copper and mercury (Beck et al., 1984). The apparent lower content of iron (less than two atoms per molecule) consistently noted in some samples of uteroferrin (see Baumbach et al., 1986) may have resulted from a partial absence of iron at the non-chromophoric site and its replacement by other metals.

A number of methods have been employed to study the iron binding sites in uteroferrin and the related beef spleen purple phosphatase. EPR, Mossbauer, NMR and magnetic susceptibility studies are consistent

with a model in which the iron atoms occupy two distinct but adjacent sites which are sufficiently close so that the two atoms are magnetically coupled (Davis and Averill, 1982; Debranner et al., 1983; Lauffer et al., 1983). Purple, enzymatically inactive uteroferrin is believed to contain two ferric ions. This form of uteroferrin gives no EPR signal, presumably because the spins of the two ferric ions are antiferromagnetically coupled, with a net spin of zero. When purple (Emax=545nm) uteroferrin is treated with mild reducing agents and turns pink (Emax=508nm), it contains a ferric-ferrous iron pair, which gives rise to an intense g'=1.74 EPR signal at liquid helium temperatures (Antanaitis and Aisen, 1983). The naturally occurring, high molecular weight pink form of uteroferrin gives rise to the same g'=1.74 EPR signal without the addition of reducing agents (Baumbach et al., 1986).

At present there is no adequate model to describe the iron binding sites of uteroferrin. Resonance-Raman studies have shown that the purple-pink color most likely arises from coordination of iron to tyrosine residues (Gaber et al., 1979). The iron atom involved is probably the less labile one at the Fe(III) site on pink reduced uteroferrin, since the molar extinction coefficient does not change when the protein is reduced from its purple to its pink form. However, the color change from purple to pink most likely represents disulfide reduction rather than reduction of iron (Schlosnagle et al., 1976). Histidine has also been implicated as a ligand for the iron atoms (Lauffer et al., 1983), and an oxygen atom has been suggested to bridge the iron sites (Mockler et al., 1983). Orthophosphate is coordinated with the iron center on purple uteroferrin (Antanaitis and Aisen, 1983).

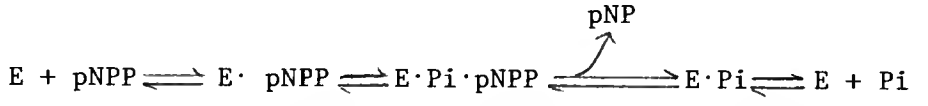
The amino acid sequence of uteroferrin revealed that the iron binding sites must be non-identical, because the protein contains no internal repeats with histidine and tyrosine residues in identical spacial relationships. It is not clear which of the 10 histidine and 10 tyrosine residues which are conserved in uteroferrin and the beef spleen enzyme could be ligands for the metals (Hunt et al., 1987).

A Proposed Reaction Mechanism for Uteroferrin

Unlike the alkaline phosphatases where the role of the Zn(II) is fairly well understood, the function of the iron atoms in catalytic activity of uteroferrin and the other purple acid phosphatases is unknown. Competitive inhibitors such as phosphate and arsenate interact with the paramagnetic centers on these proteins, which suggest that the metal contributes to substrate binding (Davis and Averill, 1982, Kawabe et al., 1984). Such substrate-iron complexes would probably be stable around pH 5.0, and may explain the acid pH optimum. The iron, by virtue of its strong electron withdrawing properties, could therefore promote catalysis.

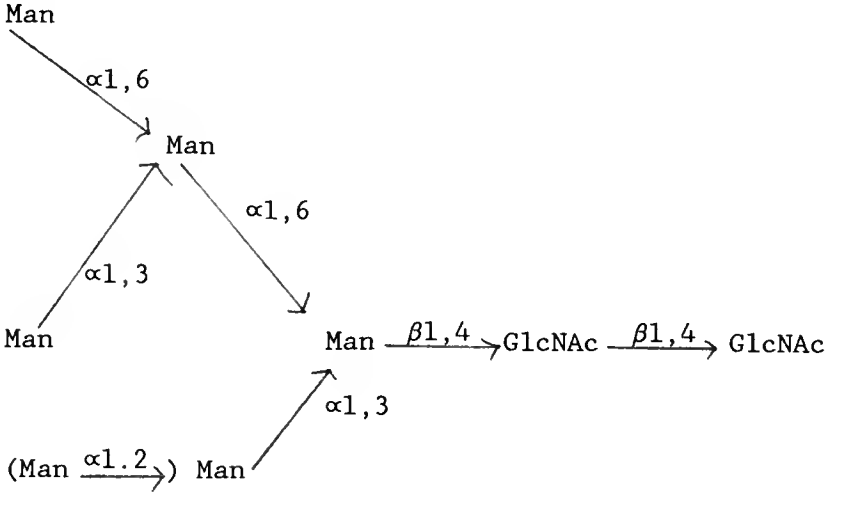
It is well documented that the reaction mechanism of the zinc-containing alkaline phosphatases involves a phosphoryl-enzyme intermediate (Levine et al., 1969). Previous experimental evidence obtained with a variety of acid phosphatases also indicated the formation of a phosphoryl-enzyme intermediate, with a double displacement reaction mechanism (Hickey and VanEtten, 1972; Igarashi et al., 1970; Feldman and Butler, 1969). Kinetic evaluation of bovine purple acid phosphatases indicated that these uteroferrin-like enzymes undergo a pseudo Uni Bi hydrolytic transfer reaction mechanism (Lau et

al., 1987; Davis et al., 1981). This theory is supported by the fact that transition state analogs of phosphate such as vanadate, molybdate and fluoride are potent inhibitors of uteroferrin and the other purple, iron-containing acid phosphatases. Lack of inhibition by p-nitrophenol suggests that the hydrolysis reaction proceeds via sequential release of p-nitrophenol and phosphate (Davis et al., 1981):



Uteroferrin Contains at Least One High Mannose Oligosaccharide

Chen et al. (1973) reported that the progesterone-induced purple protein of the porcine uterus was a glycoprotein, and that the oligosaccharide contained significant amounts of mannose. The structure of the triantennary 5 or 6 mannose oligosaccharide has been determined (Saunders et al., 1985), for uteroferrin purified from allantoic fluid and uterine flushes from pseudopregnant animals.



The biosynthesis of uteroferrin's oligosaccharide chain has been studied in vitro with explants of uterine endometrium. When [^{32}P] was provided to such cultures, the label became incorporated into the high

mannose oligosaccharide chains of uteroferrin (Baumbach et al., 1984). Approximately one-third of the oligosaccharide chains cleaved from uteroferrin by endoglycosidase H had phosphorylated carbohydrate groups: Thus, uteroferrin, a secreted acid phosphatase, carries the lysosomal recognition marker, mannose 6-phosphate, considered to be responsible for the intracellular targeting of acid hydrolyses from the Golgi complex to the lysosomes (Shephard et al., 1983). These results implied that uteroferrin is a substrate in vivo for the first of the two enzymes responsible for the addition of phosphate to terminal mannosyl residues on lysosomal enzymes. UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine 1-phosphate transferase (phosphotransferase) adds N-acetylglucosamine 1-phosphate from the nucleotide sugar UDP-N-acetyl-glucosamine to the 6-position of terminal mannose residues. Then N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (phosphodiesterase) cleaves the resulting phosphodiester bond adjacent to the N-acetylglucosamine residue (Goldberg and Kornfeld, 1983). Receptors for mannose 6-phosphate then transport these enzymes from the Golgi to lysosomes (Shephard et al., 1983). It was also demonstrated that uteroferrin is an excellent substrate for the phosphotransferase in vitro (Lang et al., 1984), in fact, it was the best substrate of those tested so far.

Why is uteroferrin secreted rather than directed to lysosomes? There are a number of possible explanations. Although uteroferrin is a good substrate for the phosphotransferase, the covering N-acetyl-glucosamine is not efficiently removed by the phosphodiesterase, thus masking the mannose 6-phosphate (Baumbach et al., 1984), and such

phosphodiesters are poor substrates for both mannose 6-phosphate receptors (Hoflack et al., 1987). The presence of only one phosphorylated mannose residue on uteroferrin's oligosaccharide may also make it a poor ligand for the mannose 6-phosphate receptors (S. Kornfeld, personal communication). The most likely explanation, however, is that the rate of uteroferrin synthesis in the pregnant uterus outstrips the intracellular targeting mechanism. Finally, it is also possible that the primary amino acid sequence of uteroferrin contains targeting information that directs it to secretory granules despite the fact that it carries phosphorylated mannose residues.

The Function of Uteroferrin

Roberts and Bazer (1980) have proposed that the primary function of uteroferrin is not phosphate ester hydrolysis, but in iron metabolism. Uteroferrin's high Km, unusual substrate specificity and lack of activity at the pH of the fluid environment in which it is found argues against an enzymatic role. The uterus of a mid-pregnant pig can produce more than two grams of uteroferrin per day (Basha et al., 1979), an amount far exceeding that which could conceivably be used for enzymatic purposes. Since large quantities of uteroferrin are known to cross the pig placenta, and the iron from [59 Fe] labeled uteroferrin is used for fetal erythropoiesis (Buhi et al., 1982a; Ducsay et al., 1982; Renegar et al., 1982), the role of uteroferrin in the pregnant pig is proposed to be in iron transport.

The pig has a non-invasive type of placentation, and at no time during pregnancy is the uterine epithelium eroded. The conceptus therefore relies upon the secretion of macromolecular products by the

mother for a large part of pregnancy. Using a variety of immunochemical and radiotracer techniques, the path of uteroferrin movement from the mother to the fetus has been determined in the pig (Buhi et al., 1982a; Ducsay et al., 1982; Renegar et al., 1982; Ducsay et al., 1984; Roberts et al., 1986b). Uteroferrin is synthesized in the uterine glands (Renegar et al., 1982), each of which is covered by a special region of the chorion (fetal placenta) called an areola. This structure is made up of specialized absorptive cells filled with large endocytotic vacuoles. Uteroferrin has been identified in these structures by immunogold staining (Roberts et al., 1986a) and within the venous drainage of the placenta. It is cleared from the bloodstream in part by the fetal liver, which is the major site of fetal hematopoiesis, and also by the kidney where it enters the urinary filtrate and is voided into the allantoic sac (Renegar et al., 1982). Uteroferrin in the allantoic fluid loses its iron to fetal transferrin, which in turn transports the iron to the fetal liver (Buhi et al., 1982a).

Uteroferrin's synthesis in the porcine uterus and its turnover in the fetal liver could theoretically supply developing fetuses with enough iron until day 75 of pregnancy. However, uteroferrin synthesis appears to fall in late pregnancy, just when iron demand for erythropoiesis is at a maximum (Roberts and Bazer, 1980). Therefore, other mechanisms of iron transport may be necessary in order to supply these needs.

Other Purple, Iron-containing Acid Phosphatases

Several other tartrate-resistant acid phosphatases have been purified from a variety of sources. A uteroferrin-like purple acid

phosphatase has been purified from the uterine secretions of mares in prolonged diestrus (Zavy et al., 1978; McDowell et al., 1982). An acid phosphatase has also been partially purified from the uterine secretions of cows treated with progesterone (Dixon and Gibbons, 1979). These discoveries were not unexpected, because the pig, the cow and the mare have similar types of non-invasive placentation.

In 1960, a basic, purple phosphoprotein phosphatase was identified in beef spleen (Revel and Racker, 1960; Glomset and Porath, 1960) and was later shown to contain iron (Campbell and Zerner, 1973). Like uteroferrin, this enzyme showed intense absorption in the range 510-545nm, and contained two iron binding sites (Davis et al., 1981). Davis et al. also demonstrated that the substrate specificities, sensitivity to inhibitors and activation by reducing agents exhibited by the beef spleen enzyme were similar to those of uteroferrin. However, the beef spleen enzyme (Mr=40,000) was reported to be made up of two subunits (Mr=24,000 and 15,000), while uteroferrin is a monomeric enzyme (Mr= 35,000). The beef spleen enzyme has been well characterized with regard to its iron binding sites, which are virtually identical to those of uteroferrin (discussed earlier). Thus, the bovine spleen enzyme's iron cluster is an asymmetrical complex containing two Fe(III) ions, one of which is reduced to Fe(II) by reductive activation of the enzymes (Davis and Averill, 1982; Antanaitis and Aisen, 1983; Debranner et al., 1983; Antanaitis and Aisen, 1982).

The function of the beef spleen enzyme has been addressed only recently. Schindelmeiser et al. (1987) have localized the enzyme to lysosomal-like organelles of cells of the reticulo-phagocytic system.

The phagocytosing cells that contained the phosphatase were frequently found in close contact with clusters of aged and deformed erythrocytes. The authors hypothesized that the enzyme is involved either in the breakdown of erythrocyte membrane and cytoskeletal phosphoproteins, or in the metabolism of iron from these erythrocytes.

There have been reports of a bovine skeletal tartrate-resistant acid phosphatase, assumed to be distinct from the bovine spleen enzyme (Lau et al., 1985, 1987). This bovine skeletal enzyme resembles the bovine spleen enzyme and porcine uteroferrin in its molecular weight, pH optimum, substrate specificities and sensitivity to inhibitors. Like uteroferrin, and unlike the bovine spleen enzyme, the bovine skeletal enzyme is reported to be monomeric. The authors chose to stress the phosphotyrosine phosphatase activity of the enzyme and postulated that the skeletal acid phosphatase functions in the regulation of proliferation and differentiation. The authors have not yet described a physiologic substrate for this phosphoryrosyl-specific protein phosphatase, and have not demonstrated that it plays an important role in cell growth. Thus, the function of the acid phosphatase in bone remains unknown.

Purple, iron-containing acid phosphatases have been purified from rat spleen (Hara et al., 1984) and rat bone (Kato et al., 1986; Anderson and Toverud, 1986), and an immunologically related phosphatase was purified in small quantities from rat epidermis (Hara et al., 1985). These monomeric enzymes also resembled uteroferrin in molecular weight, substrate specificities and sensitivity to inhibitors. Hara et al. (1984, 1985) believe that the spleen and epidermis enzymes may play a

role in nucleotide metabolism and that the bone enzyme may be acting as a phosphoprotein phosphatase in vivo. Although Anderson and Toverud (1986) have indicated that studies were underway to determine the function of the rat bone enzyme, the functions of the rat enzymes also remain obscure.

Human Acid Phosphatases

The human acid phosphatases were first characterized by researchers interested in the clinical significance of acid phosphatase levels in normal and pathological tissue. One of the human enzymes, a cationic, tartrate-resistant enzyme known as the Type 5 phosphatase, was of interest to us because it seemed as though it might be related to the well characterized abundant acid phosphatase, porcine uteroferrin.

Characterization of the Human Acid Phosphatases

Li et al. (1970) described electrophoretic separation of human leukocyte acid phosphatases on 7.5% polyacrylamide gels at pH 4.0, which were then stained for acid phosphatase activity. They detected six isozymes of acid phosphatase, varying in electrophoretic mobility. Band 0 did not enter the gel. Band 1 was the slowest migrating form, band 5 the fastest and most basic. The various isozymes could also be distinguished by their substrate specificities and sensitivity to inhibitors.

Lam et al. (1973) described in detail the distribution of acid phosphatases in normal human tissue. Isozymes 1 and 3 were the most widely distributed and most active forms. Higher type 2 levels were characteristic of the prostate. Type 5 was the least common and least

abundant isozyme, detected in only small quantities in spleen, kidney and liver.

These researchers noticed differences in acid phosphatase levels and in relative isozyme ratios in normal plasma and leukocytes versus those from patients with certain diseases. In normal leukocytes, band 1 was the strongest, bands 2, 3 and 4 were of moderate strength and band 5 was very weak (Yam et al., 1971). Patients with infectious mononucleosis, for example, exhibited elevated levels of isozymes 1, 3 and 5 in leukocytes (Li et al., 1973). Isozyme 2 was found to be elevated in the plasma of patients with prostatic cancer (Lam et al., 1973). Perhaps the most striking isozyme pattern was evident in the leukocytes and spleen homogenate (but not plasma) of patients with hairy cell leukemia. The leukemic white cells of these patients produced very small amounts of isozymes 1 through 4, and large quantities of isozyme 5 (Yam et al., 1971).

Yam et al. (1971) also determined that while acid phosphatase isozymes 1,2,3 and 4 were completely inhibited by 50mM L-(+)-tartrate, isozyme 5 was unaffected by this reagent. (Recall that uteroferrin is resistant to inhibition by tartrate). This observation made it easier to detect the presence of elevated levels of the Type 5 isozyme in hairy cell leukemia and allowed cytochemical localization of only this isozyme in leukocytes and tissue sections. The presence of tartrate-resistant acid phosphatase activity in the hairy cells in blood smears is the major method of diagnosis of hairy cell leukemia today (Nanba et al., 1977; Janckila et al., 1978; Braylan et al., 1979).

Hairy Cell Leukemia

The origin of hairy cells has been a subject of controversy for a number of years. Surface marker studies done with monoclonal antibodies have revealed hairy cells with properties common to both B-cells and T-cells (Burns et al., 1980; Worman et al., 1983; Armitage et al., 1985). Hairy cells have been shown to phagocytose latex particles, properties which indicated a common origin with monocytes or histiocytes (Rosenszajn et al., 1976). However, most researchers now agree that hairy cells have originated from B-lymphocytes, possibly splenic white pulp marginal zone lymphocytes (Van der Oord et al., 1985). More specifically, hairy cells may originate from a lymphocyte in a terminal stage of differentiation, at the point of switching from an IgM-bearing small lymphocyte to a mature plasma cell (Jansen et al., 1979). Recently it was proposed that the hairy cell may represent the terminally differentiated B-lymphocyte (Robinson et al., 1985; Gazitt and Polliack, 1987).

Clinically, hairy cell leukemia is represented by a slow insidious onset, splenomegaly, anemia and the appearance of leukemic hairy cells in the spleen, bone marrow and peripheral blood (Nanba et al., 1977). Significant quantities of tartrate-resistant acid phosphatase are contained in the reticulum and epithelioid cells of the patients' spleens. In the past splenectomy was often performed in order to reduce the tumor load. More recently, it has been demonstrated that α-interferon treatment is successful in causing remission in hairy cell leukemia patients (see Porzsolt, 1986).

Tartrate-resistant Acid Phosphatase Levels in Other Diseases

Tartrate-resistant acid phosphatase levels are elevated in several types of leukemia other than hairy cell leukemia but not in a consistent manner. Drexler et al. (1985, 1986) have studied acid phosphatase levels in a variety of human leukemia cell lines and in fresh leukemia cells obtained directly from patients. These researchers found that the few leukemia cell lines which did express the phosphatase were those which represented lymphocytes arrested late in differentiation. Many researchers now believe that tartrate-resistant acid phosphatase may be useful as an enzymatic marker for B-lymphocyte differentiation (Drexler et al., 1985) and that measurement of its activity may have prognostic value (Pieters and Veerman, 1987).

Measurement of serum acid phosphatase levels may also be of clinical significance. While the Type 5, tartrate resistant acid phosphatase is not detected at high levels in the plasma of patients with hairy cell leukemia, plasma levels of this isozyme are high in patients with diseases which involve bone reabsorption and regeneration, and in patients with malignancies metastasized to bone (Lam et al., 1978). These diseases include Paget's disease, which is a type of bone tumor, and osteoporosis (Li et al., 1973). Tartrate-resistant acid phosphatase activity is also elevated in the plasma of children during bone growth (Chen et al., 1979). Plasma levels of the Type 5 phosphatase are also high in patients with Gaucher's disease, which is an inherited glycosphingolipid storage disease characterized by β-glucocerebrosidase deficiency. The phosphatase in Gaucher's disease is believed by some

researchers to be of osteoclast origin, and most likely a secondary phenomenon indicative of bone involvement (Choy, 1985).

It is interesting to note that while the acid phosphatase of hairy cells is intracellular, confined to discrete inclusion bodies resembling lysosomes (Lam et al., 1976), osteoclasts secrete this enzyme under the influence of parathyroid hormone (Miller, 1985; Braidman et al., 1986; Chambers et al., 1987). It is not clear whether the secreted osteoclast and lysosomal spleen Type 5 phosphatases are distinct enzymes.

Purification and Characterization of the Human Type 5 Isozyme

In all of the studies described above, acid phosphatase levels were measured by histochemical methods or by enzymatic staining of non-denaturing polyacrylamide gels. There are a few reports in the literature of attempts to purify and characterize the human tartrate-resistant acid phosphatase. Although these reports contain some contradictory information, one fact becomes apparent: This human Type 5, tartrate-resistant acid phosphatase has a number of properties reminiscent of porcine uteroferrin.

An attempt was made by Lam and Yam (1977) to purify this human Type 5 acid phosphatase from the spleen of a patient with hairy cell leukemia. They were not able to purify the enzyme to homogeneity. Although the authors reported an Mr of 64,000, which is almost twice that of uteroferrin, many of the characteristics they reported for the enzyme were reminiscent of the characteristics of uteroferrin and the other purple, iron-containing acid phosphatases. The human hairy cell enzyme, like uteroferrin, was a very basic protein. This human enzyme,

also like uteroferrin, hydrolyzed p-nitrophenylphosphate, ATP and pyrophosphate but was virtually inactive towards hexose phosphates, β-glycerol phosphate and AMP. The human enzyme was also activated by mild reducing agents and very sensitive to inhibition by molybdate.

A tartrate-resistant acid phosphatase was also purified from a Gaucher's disease spleen (Robinson and Glew, 1980; 1981). This enzyme was believed to be distinct from the hairy cell spleen enzyme. Like uteroferrin and the other purple, iron-containing acid phosphatases, this enzyme was reported to have an Mr of 33,000. It bound avidly to Concanavalin A Sepharose, indicating its glycoprotein nature. It, too, was sensitive to inhibition by molybdate, fluoride and dithionite. However, this enzyme had a number of features which were unlike the hairy cell enzyme. It was reported that the Gaucher enzyme was made up of two subunits of Mr 20,000 and 16,000, did not hydrolyze pyrophosphate, and was not activated by reducing agents. Thus, it was not clear whether the Gaucher spleen and hairy cell spleen enzymes were the same protein. Efstratiatis and Moss (1985) partially purified an Mr=37,000 tartrate-resistant, molybdate-sensitive acid phosphatase from lung, and detected an apparently identical enzyme in osteoclasts. It is not known whether the lung/osteoclast tartrate-resistant acid phosphatase is identical to either of the spleen enzymes.

Although it was once believed that uteroferrin was uniquely associated with the uterus (Roberts and Bazer, 1980), it is now clear that there is a growing family of purple, iron-containing acid phosphatases. The human tartrate-resistant acid phosphatase may belong to this enzyme family. Because of the clinical significance of the

human Type 5 phosphatase, it seemed to be a subject worthy of further study. In Chapter 2, the purification of the human tartrate-resistant acid phosphatase to homogeneity is described, and its physical properties are clearly defined. The enzymatic characteristics of the phosphatase are described in detail, and the immunologic relationship of the human enzyme to the purple, iron-containing acid phosphatases is investigated. Chapter 3 deals with the molecular cloning of a cDNA coding for the human tartrate-resistant acid phosphatase, the characteristics of the deduced amino acid sequence, and the expression of the enzyme in leukemia cells.

CHAPTER 2
THE TYPE 5, TARTRATE-RESISTANT ACID PHOSPHATASE FROM SPLEEN OF HUMANS WITH HAIRY CELL LEUKEMIA. PURIFICATION, PROPERTIES, IMMUNOLOGICAL CHARACTERIZATION, AND COMPARISON WITH PORCINE UTEROFERRIN.

Introduction

Measurement of serum and tissue levels of acid phosphatase can prove useful in the diagnosis of several human disease states (Gutman et al., 1936; Yam et al., 1980; Drexler and Gaedicke, 1983; Yam et al., 1983; Allhoff et al., 1983). At least six isozymes of acid phosphatase have been identified by their relative electrophoretic mobilities towards the cathode at low pH. The most basic, a minor isozyme known as Type 5, is elevated in the circulating white cells and enlarged spleens of patients with hairy cell leukemia (Lam et al., 1980; Yam et al., 1983; Yam et al., 1971; Lam and Yam, 1977) and in spleens and sera of patients with Gaucher's disease (Choy, 1983). This same isozyme also appears to be a secretory product of osteoclasts (Minkin, 1982; Chen et al., 1979; Stepan et al., 1983), and of osteoclastic bone tumors (Chen et al., 1979; Tavassoli et al., 1980). The Type 5 phosphatase can be distinguished from the other isozymes, not only by its basic nature, but also because it is not inhibited by L-(+)-tartrate. Indeed, the cytohistochemical demonstration of a tartrate-resistant acid phosphatase within leukemic hairy cells is currently the most usual method of diagnosing this rare form of leukemia (Nanba et al., 1977; Janckila et al., 1978; Braylan et al., 1979).

The enzyme produced by the spleen histiocytes of patients with Gaucher's disease has been purified in very small quantities by Robinson and Glew (1980). This phosphatase was activated by reducing agents and had high catalytic activity towards a variety of substrates, both artificial and natural, which had strong leaving groups, but had very low activity towards aliphatic phosphate esters. It was also a phosphoprotein phosphatase. A similar enzyme has been isolated from the spleen of a patient with hairy cell leukemia (Lam and Yam, 1977). However, the latter enzyme appeared to differ from the Gaucher phosphatase in a number of respects, including its response to reducing agents, its molecular weight and its substrate specificity. Moreover its specific activity was about 10-fold lower than that of the Gaucher enzyme.

The enzymatic properties of the Type 5 human isozyme are very similar to those of a well characterized, abundant acid phosphatase known as uteroferrin. This unusual molecule is a purple colored, iron-containing glycoprotein that was first purified from uterine secretions of pigs (Chen et al., 1973). Its synthesis, which occurs within the glandular epithelium of the uterus (Renegar et al., 1982), is under progesterone control (Roberts and Bazer, 1980). During pregnancy approximately 2g of the protein are produced daily (Basha et al., 1979). It has been shown to be taken up by the overlying placenta and to enter the fetal blood stream (Renegar et al., 1982). Its iron is then rapidly utilized in fetal erythropoiesis (Ducsay et al., 1982; Buhi et al., 1982a). A role for uteroferrin in transplacental iron transport has been proposed (Roberts and Bazer, 1980; Buhi et al., 1982a). However,

the fact that uteroferrin, particularly when activated with mild reducing agents such as ascorbate or mercaptoethanol, is a potent acid phosphatase (Schlosnagle et al., 1974; 1976) has raised questions as to whether iron transport is the major or only function of the protein (Davis et al., 1981). In this regard, acid phosphatases which are clearly similar to uteroferrin in their spectral properties, molecular weight, and iron content have been isolated from a variety of sources (Antanaitis and Aisen, 1983). In addition, there are several descriptions of acid phosphatases, including the Type 5 human isozyme, which resemble uteroferrin in their enzymatic properties, but which have either not been purified or else have been isolated in such small quantities that they have not been fully characterized (Roberts and Bazer, 1976). For the above reasons it has been proposed that uteroferrin may be but one member of a broad class of acid phosphatases which has a wide distribution (Roberts and Bazer, 1980; 1976).

Chapter 2 contains the description of a simple method for the purification of the enzyme from human spleen by making use of antibodies to the readily available uteroferrin. It is demonstrated conclusively that this human isozyme belongs to the class of iron-containing acid phosphatase best illustrated by uteroferrin. Monoclonal antibodies have been prepared against both uteroferrin and the human enzyme which appear to have broad reactivity towards this class of enzyme.

Materials and Methods

Materials

CM-cellulose was obtained from Whatman, Sephadex G-100 and Sepharose-4B from Pharmacia, and Triton X-100 from Rohm and Haas Co.

All substrates, activators, and inhibitors (except anions) used for acid phosphatase assays, cyanogen bromide, protein standards, Fast Garnet GBC salt, buffers and protease inhibitors were purchased from Sigma. Reagents for the Bradford protein assay were purchased from Bio-Rad. Isozyme standards from serum (for human acid phosphatase activity) were obtained from Calbiochem-Behring. All other chemicals (reagent grade or better) were obtained from Fisher.

Methods

Purification of uteroferrin. Uteroferrin was purified from the uterine secretions of pseudopregnant pigs or from allantoic fluid as previously described (Schosnagle et al., 1974; Roberts and Bazer 1980). Allantoic fluid was dialyzed overnight at 4°C against 0.01M Tris-HCl, pH 8.2. A slurry of carboxymethyl-cellulose (CM-cellulose; 100ml of settled resin per 4L allantoic fluid) was added in the same buffer. This mixture was stirred for 1 hour, then packed into a column and washed with the above buffer. The bound, basic proteins were eluted in one step by a high salt buffer (0.01M Tris-HCl, pH 8.2, 0.5M NaCl). The proteins were dialyzed against 0.01M Tris-HCl, pH 8.2 and applied to a Sephadex G-100 column (5 x 90 cm) which had been previously equilibrated in 0.1M sodium acetate, pH 4.9 and 0.33M NaCl. (Uterine secretions of pseudopregnant animals were loaded directly onto the Sephadex G-100 column after centrifugation at 10,000xg at 4°C for 30 minutes.) The pink, high molecular weight uteroferrin peak, which immediately preceded the purple peak, was pooled and used without further purification. The uteroferrin peak, purple in color, was pooled and dialyzed against 0.01M Tris-HCl, pH 8.2. The uteroferrin was then loaded onto a column of

CM-cellulose and eluted with a linear salt gradient (0.01-0.5M NaCl). Uteroferrin eluted as a symmetrical peak between 0.20 and 0.25M NaCl. The final preparations of uteroferrin had A_{280}/A_{545} ratios of less than 14 and gave a single band of apparent Mr=35,000 upon polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS).

Purification of acid phosphatase from human spleen. Spleen tissue was used from a male aged 52 who was diagnosed as having hairy cell leukemia. The weight of the spleen was 2.3kg. The normal control spleen from an accident victim weighed 0.22kg. Tissue was cut into slices, the outer fascia removed, and the slices diced into small cubes. These were homogenized at 4°C in extraction buffer [0.05M Tris acetate, pH 7.5, 2% (v/v) Nonidet P-40, 0.02% (w/v) sodium azide, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM EDTA, and aprotinin (1 trypsin inhibitory unit/ml)] by means of a Teckmar Model SDT homogenizer (half-full setting) with the largest probe until a homogeneous preparation was obtained. A volume of 150ml of extraction buffer was used for each 100g of tissue. The homogenate was centrifuged (30,000xg; 20 minutes) and the supernatant fraction dialyzed overnight against 0.01M Tris-HCl buffer, pH 8.2. A slurry (50ml) of CM-cellulose was added to the retentate and stirred for 1 hour. This material was collected on a Whatman No. 4 filter paper on a Buchner funnel. After it was washed several times with 0.01M Tris-HCl buffer, pH 8.2, the CM-cellulose was suspended in the same buffer and loaded into a glass column. Proteins which had remained bound were eluted with 0.5M NaCl. In initial experiments, the eluted material was dialyzed and loaded onto a column of CM-cellulose (1 x 5cm) in 0.01M Tris-HCl buffer, pH 8.2.

Elution was performed by means of a gradient (180ml; 0.01-0.5M) of NaCl at pH 8.2. Fractions which contained the enzyme were pooled, dialyzed, and freeze-dried, and then subjected to gel filtration on a column (2.5 x 80cm) of Sephadex G-100 which was equilibrated with 0.01M sodium acetate buffer, pH 4.9, containing 0.33M NaCl. Elution of the protein was followed by its absorbance at 280nm and by measuring acid phosphatase activity (see below). Fractions which contained the phosphatase were pooled and loaded directly onto an anti-uteroferrin immunoaffinity column (see below).

After the immunoaffinty column had been washed thoroughly with loading buffer (0.01M Tris-HCl, pH 8.2, containing 0.3M NaCl), the bound enzyme was eluted with 0.05M glycine-HCl buffer containing 0.15M NaCl, pH 2.3. It was collected in 1ml fractions. Each fraction was immediately neutralized with 0.1ml of 1M Tris-HCl, pH 8.2.

More recently, enzyme which had been eluted form the CM-cellulose column with 0.5M NaCl was loaded directly onto the immunoaffinity column without including intervening ion exchange and gel filtration steps.

Purification of acid phosphatase from human placenta. A normal term placenta, weighing 630g was employed to purify the acid phosphatase by exactly the same procedure described for human spleen. An alternative method of enzyme purification was employed for a second normal term placenta weighing 650g. The second placenta was homogenized in 1300ml of a lysis buffer which consisted of 0.3M KCl, 2% (v/v) Nonidet P-40, 1mM PMSF and 0.2% (w/v) sodium azide. After centrifugation at 30,000xg for 20 minutes at 4°C, protamine sulfate was added to the supernatant fraction to 0.1% (w/v). The solution was stirred for 1 hour at 4°C,

then centrifuged at 30,000xg for 15 minutes. The resulting supernatant fraction was dialyzed overnight against 0.01M Tris-HCl, pH 8.2. A slurry of CM-cellulose (50ml) was added and the mixture stirred for 1 hour at 4°C. The bound proteins were eluted from the CM-cellulose as described for uteroferrin. The entire pool of basic proteins was then subjected to immunoaffinity chromatography as described for the human spleen enzyme.

Purification of the bovine spleen acid phosphatase. The procedure described for the human spleen enzyme was also employed for the bovine spleen enzyme. Approximately 200g of beef spleen tissue were used.

Purification of the rat spleen phosphatase. The spleens of three rats (approximately 2g) were homogenized as described for the human enzyme. After centrifugation at 30,000xg, the supernatant fraction was loaded directly onto the immunoaffinity column. Elution was performed as described for the human enzyme.

Purification of the bovine uterine acid phosphatase. Bovine uterine fluids were obtained from the ligated uterine horn of day 270 unilaterally pregnant cattle (Bartol, 1983). Approximately 400ml of bovine uterine fluids were dialyzed overnight against 4 liters of 0.01M Tris-HCl, pH 8.2. The solution was centrifuged (30,000xg; 20 minutes) to remove particulate matter and passed through a column (1 x 5cm) of CM-cellulose equilibrated in the same buffer. After washing the column, proteins which had bound were eluted with 0.5M NaCl in 0.01M Tris-HCl, pH 8.2. These basic proteins were then subjected to gel filtration on a column (2.5 x 80cm) of Sephadex G-100 which had been equilibrated with 0.01M acetate buffer, pH 4.9 containing 0.33M NaCl. Protein

concentration was monitored by absorbance at 280nm, and acid phosphatase activity monitored by p-nitrophenylphosphatase activity (see below).

Measurement of p-nitrophenylphosphatase activity. The standard colorimetric assay utilized an appropriately diluted sample of enzyme, 20mM p-nitrophenylphosphate and 0.1M sodium acetate buffer (pH 4.9 for uteroferrin, pH 5.3 for the human and rat enzymes, pH 6.0 for the beef spleen enzyme and pH 4.5 for the bovine uterine enzyme) in a final volume of 1ml. The enzymes were assayed in the presence of 0.1M 2-mercaptoethanol except where stated in the text. The reaction was allowed to proceed at 37°C for 10 minutes and stopped by the addition of 1ml of 1M NaOH. The absorbance was then read at 410nm. Activity is expressed as units, where one unit is the release of 1μmol p-nitrophenol/minute.

Hydrolysis of other substrates. With the exception of phosvitin, other substrates were assayed at a final concentration of 5mM. The release of orthophosphate was measured spectrophotometrically by the method of Bartlett (1959), which involves the reduction of phosphomolybdate to a blue color (Emax=830nm). Blanks without enzyme were run for each substrate. The release of orthophosphate from phosvitin (10mg/ml) was measured as described by Roberts and Bazer (1976).

pH optima. The pH optima of the acid phosphatases were determined with the use of 0.1M sodium acetate, pH 3.0-5.4, 0.1M Tris acetate, pH 5.6-6.5, and 0.1M Tris-HCl, pH 7.0-7.5 as buffers. The substrate used was p-nitrophenylphosphate.

Protein determination. Protein concentration was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard. In order to measure low amounts of protein (less than 10μg) the method of Bradford (1976) was employed with lysozyme as a standard (see Bio-Rad Bulletin 1069, February 1979).

Determination of iron. Iron was measured by the method of Cameron (1965) as modified by Campbell and Zerner (1973). All glassware was soaked in 4M HCl and rinsed several times with iron-free water to remove metal contaminants. Protein samples, blanks and ferrous ammonium sulfate standards were treated with 70% (v/v) perchloric acid for 30 minutes at room temperature to release iron from the protein. A solution of 10% (w/v) hydroxylamine hydrochloride was added, and the samples were left at room temperature for 30 minutes. Finally, batho-phenantroline solution (4mg/ml), pyridine, and iron free water were added successively to each sample and the solutions mixed thoroughly. The absorbance of the solutions was measured at 536nm. The concentration of iron in the protein was obtained from a plot of absorbance versus iron concentration of the standards, which was linear over a range of 0-4 μg.

Immunoaffinity chromatography. Sepharose CL-4B, purchased in its cyanogen bromide activated form, was prepared according to the instructions of the manufacturer. Protein was coupled to the matrix in 0.1M sodium carbonate, pH 8.5 and 0.5M NaCl at 4°C on a tube turner. Alternatively, Sepharose 4B was activated for 30 minutes at 0°C in 1M potassium phosphate, pH 12, using cyanogen bromide (Cuatrecacas et al., 1968) dissolved in N', N' dimethyl formamide. The gel was washed with

0.1M sodium bicarbonate, pH 8.9. Proteins were coupled at 4°C overnight on a tube turner in a buffer of 0.1M sodium bicarbonate, pH 8.9 and 0.5M NaCl. Unreacted sites on the matrices were blocked with 1M ethanolamine, pH 8.9 for 4 hours at 4°C, after washing out unbound protein with the coupling buffer. Finally, the gel was washed with 0.05M sodium acetate, pH 4.0, plus 1M NaCl, followed by 0.05M sodium borate, pH 8.9, plus 1M NaCl.

Uteroferrin (10mg/ml) purified as described above was coupled to the matrix with a 30-50% coupling efficiency. When stored at 4°C in the presence of 0.1% (w/v) sodium azide, it retained its purple coloration and acid phosphatase activity for over a year. The uteroferrin-Sepharose matrix was employed to purify anti-uteroferrin polyclonal antibodies (see below). Anti-uteroferrin polyclonal antibodies were then coupled to the matrix at a concentration of 10mg protein per ml of gel. This anti-uteroferrin immunoaffinity matrix was employed to bind the various spleen enzymes.

Preparation of polyclonal antibodies against uteroferrin. Samples of uteroferrin (up to 10mg) were mixed with 5ml of Freund's Complete Adjuvant to form an emulsion and injected intradermally over the shoulder, rear leg and abdominal regions of one side of a female lamb. Seven days later the procedure was repeated on the other side of the lamb. A third injection was given seven days after the second. One month after the first injection, serum was collected by jugular venipuncture. The serum was allowed to clot at 4°C overnight. Following centrifugation at 1000xg for 20 minutes, the serum was frozen in small aliquots.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% (w/v) gels according to the method of Laemmli (1970). Protein samples were solubilized in 5% (w/v) SDS, 5mM Tris-HCl, pH 6.8, and 5% (v/v) 2-mercaptoethanol. Gels were stained with 0.125% (w/v) Coomassie Brilliant Blue.

Electrophoresis was carried out under non-denaturing conditions according to the method of Reisfield et al. (1962) as modified by Basha et al. (1979). Basic proteins were resuspended in 5mM Tris-HCl, pH 6.8 and 10% (v/v) glycerol. The samples were subjected to electrophoresis in 7.5% (w/v) polyacrylamide gels, pH 4.0 (which did not contain SDS) in a β-alanine buffer, pH 4.5. Proteins with acid phosphatase activity were visualized with α-naphthylphosphate substrate and fast Garnet GBC as the coupling dye.

Binding to Concanavalin A-Sepharose (Con A-Sepharose). Enzyme samples in 0.3M NaCl were adjusted to pH 6.5 and loaded onto a column of Con A-Sepharose (approximately 5ml bed volume). The column was washed with 3 volumes of 0.1M Tris-HCl, pH 8.2, 0.3M NaCl, 1mM $CaCl_2$, 1mM MnCl and 0.02% (w/v) sodium azide. Bound protein was eluted with warm (50-60°C) 0.01M α-methyl-D-mannoside or 0.1M acetic acid (Baumbach et al., 1984).

Immunization of mice. Female Balb/c mice at 8-48 weeks of age were injected with 17.5μg of antigen intraperitoneally (i.p.; Katzmann et al., 1981) with antigen emulsified in 100μl Freund's Complete Adjuvant. A subsequent injection of 17.5 μg was performed as above with Freund's Incomplete Adjuvant, 10 days later. On day 17, a final injection was administered in normal saline (0.9%, w/v) via the tail vein. The

antigens employed were as follows: For fusion 5, high molecular weight uteroferrin was employed. This fusion was outlined in detail by Baumbach (1984) and was carried out in collaboration with George Baumbach. For fusion 6, uteroferrin (purple, Mr=35,000) was employed. For fusion 13 the human hairy cell acid phosphatase was used.

Generation of monoclonal antibodies. The SP2/0-Ag 14 (SP2/0; Schulman et al., 1978) non-secreting myeloma cell line was cultured in Dulbecco's Modified Eagle's medium (DME) containing 10% (v/v) each of heat inactivated fetal calf serum and agammaglobulinemic horse serum; antibiotic solution (10μg/ml penicillin, 10μg/ml streptomycin,0.25μg/ml amphotericin B), and 2mM glutamine. One day-old (conditioned) medium from logarithmic cultures was harvested and used for hybridoma medium supplementation.

The method of splenocyte fusion and propagation of the resulting hybrid cells was by established methodologies (Kennet et al., 1980; Oi and Herzenberg, 1980). Two mice were anaesthetized with ether and the spleens excised aseptically, trimmed of fat and then minced with sterile scalpel blades in 20ml of DME. The tissue was then triturated several times and the large debris allowed to settle. The splenocytes in the supernatant fluid were then collected by centrifugation at 1000xg for 5 minutes at room temperature, washed in 15ml of DME, and resuspended in a final volume of 5ml DME and kept at room temperature. Approximately 10^8 SP2/0 cells were mixed and centrifuged at 1000xg for 5 minutes. The mixture of cells was resuspended in 1ml of 50% (w/v) polyethylene glycol 1000 in DME for 1 minute, then diluted to 31ml by the addition of 6ml each minute for 5 minutes of DME-HAT (DME with hypoxanthine, 0.1mM;

aminopterin, 0.8μM; thymidine, 16μM; Littlefield, 1964) supplemented with 10% (v/v) each of fetal calf serum and heat-inactivated agammaglobulinemic horse serum, 40% (v/v) conditioned medium and 2mM glutamine. Aliquots (100μl) were seeded into 96 well microtiter culture dishes and incubated in an atmosphere of 5% CO_2 at 37°C.

Two weeks later, the culture supernatant fluid was withdrawn and assayed for specific binding to uteroferrin using a solid phase binding assay (see below). Positive cultures were resuspended in 500μl of DME-HAT supplemented as described above and placed in 48 well cluster dishes. When a culture was confluent, the cells were transferred to 25cm^2 culture flasks and maintained in the medium described for SP2/0 cells. When cultures were growing well, cell densities were counted with a hemacytometer and the cells cloned by limiting dilution (Kennet et al., 1980). The cells were diluted to yield an average of 5, 1 or 0.1 cells per 100μl. Thirty-two wells were seeded with each dilution, and the initial cell density which subsequently had less than 33% of the wells with growth was rescreened for antibody production. Hybridoma cells were maintained as described for SP2/0 cells and were frozen in the standard growth medium with 30% (v/v) fetal calf serum and 10% (v/v) glycerol or 10% (v/v) dimethyl sulfoxide.

Purification of mouse antibodies. Immune sera from mice were collected immediately prior to splenectomy by cutting the descending aorta aspirating the blood into a hypodermic syringe. Nonimmune (normal) mouse sera were collected from uninjected, healthy, anaesthetized animals.

Culture medium from growing hybridoma cells was used as a source of monoclonal antibody in some instances. In other cases ascites fluid was prepared by growing cloned hybridoma cells (1×10^7) in the peritoneal cavity 5 days after 2 i.p. injections of 0.5ml pristane (2,6,10,14-tetramethylpentadecane) spaced 10 days apart. The ascites fluid was harvested after the tumor had grown sufficiently, about 10-14 days. The sample was allowed to clot at 4°C, centrifuged at 1000xg for 20 minutes, and sodium azide was added to 0.02% (w/v).

Iodination of antibodies. Iodination was performed according to the method of Markwell and Fox (1978) and Markwell (Pierce Chemical Company bulletin, 1978). Glass tubes were coated with 100μg Iodogen (1,3,4,6-tetrachloro-3α-6α-diphenylglycolluril) by evaporation of methylene chloride. Mouse antibodies against uteroferrin or the human enzyme (100μg) in 1ml of buffer (0.02M sodium barbital, pH 7.5, 0.4M NaCl) were added to the Iodogen coated tube. Carrier-free $Na^{125}I$ (100μCi) was added and the tube shaken gently every minute for 15 minutes. The iodinated protein was separated from unreacted ^{125}I by dialysis (0.01M Tris-HCl, pH 8.2) or gel filtration on a column of Sephadex G-50 (0.01M Tris-HCl, pH 8.2, 0.2M NaCl).

Assay of antibodies. Antigen was dialyzed overnight against phosphate-buffered saline (PBS; 0.14M NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 3mM KCl, 0.5mM $MgCl_2$, 1mM $CaCl_2$, pH 7.4) and adjusted to a concentration of 50μg/ml. Antigen was passively adsorbed overnight at 4°C to polyvinylchloride microtiter flex plates as outlined by Tsu and Herzenberg (1980). The plates were washed three times with PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) sodium

azide. Hybridoma culture medium (0.05ml) or diluted mouse antisera were added and allowed to bind for 1 hour at room temperature. The plates were washed as above and 50,000cpm of ^{125}I-labeled anti-mouse IgG in 50ml were added and allowed to bind for 1 hour. The plates were washed as above, the wells cut out and bound radioactivity measured in a gamma counter. Immune and non-immune sera were replicate tested six times, and culture media were tested in duplicate.

Affinities of antibodies. Monoclonal antibodies against uteroferrin or the spleen enzyme were affinity purified from either cell growth medium or from ascites fluid on uteroferrin immobilized on Sepharose 4B. The procedure was identical to that described earlier. The purified antibodies were serially diluted in PBS containing BSA (1mg/ml) over a range of concentrations extending from 10^{-6} to 10^{-10}M. Uteroferrin or the spleen acid phosphatases were plated out into wells on flexvinyl plates as described in the previous section. After the wells were washed and the remaining adsorption sites were blocked with BSA, the plates were dried. Appropriately diluted solutions of antibody (0.05ml) were then added to the wells and after 1 hour they were withdrawn and the plates were washed. The amount of antibody that bound at each dilution was then measured by addition of a constant amount of a second labeled antibody. This antibody was ^{125}I-labeled sheep anti-mouse IgG (see below). Its specific activity was approximately 10^6 cpm/μg. After 1 hour, this solution was withdrawn, the plates were washed and each well was analyzed for radioactivity separately in a gamma counter. All determinations were carried out in duplicate. Controls were described in the previous section. The binding affinities of each monoclonal

antibody to the different antigens were calculated by the method of Scatchard (1949).

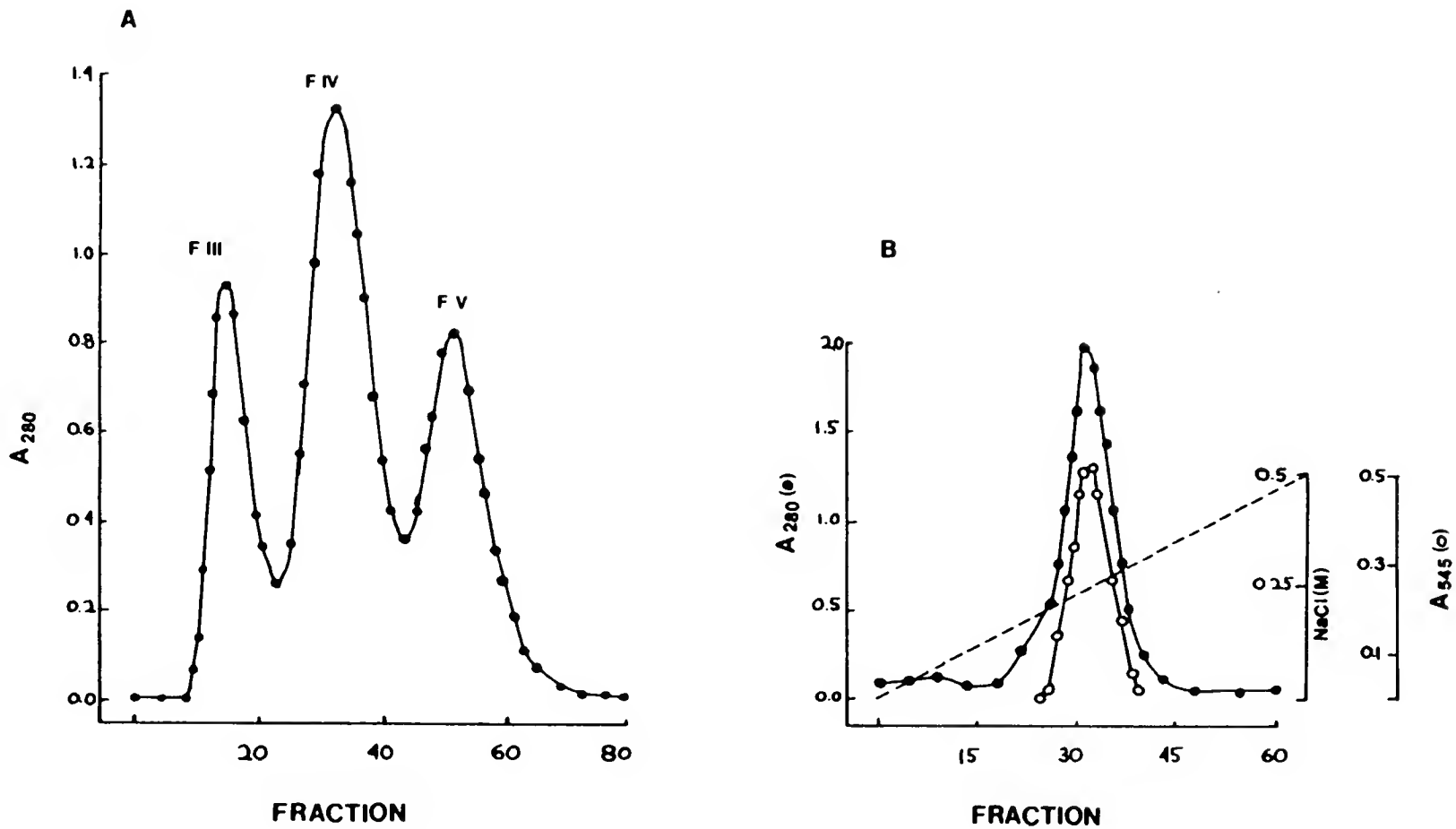
Competitive binding of monoclonal antibodies. In order to determine whether various monoclonal antibodies bound to the same or different sites on uteroferrin, a competitive binding assay was employed. Uteroferrin was adsorbed to the wells of flexvinyl plates as described earlier. Monoclonal antibodies were iodinated with ^{125}I to a specific activity of approximately 10^6 cpm/μg. Samples of antibody (0.05ml; 50,000cpm) were added to each well in presence of increasing concentrations (10^{-6}-10^{-10}M) of the second unlabeled antibody. If this second antibody failed to compete or competed poorly with the labeled antibody for binding to uteroferrin, it was assumed to occupy a distinct site on the protein. Three monoclonal antibodies (5.122.10, 6.21.2, and 6.22.1), which bound to uteroferrin with high affinity, appeared to compete only poorly with each other in the above assay.

Results

Purification of Uteroferrin

Figure 2-1A shows the fractionation on Sephadex G-100 of the basic proteins obtained from one sample (4 liters) of allantoic fluid obtained from a day 67 pregnant pig. Three major protein fractions were apparent and designated FIII, FIV and FV by previous convention (Murray et al., 1972; Chen et al., 1973). The average molecular weights of these fractions were calculated to be approximately 80,000, 35,000 and 15,000 respectively. Fraction V contained lysozyme (Roberts et al., 1976) and a group of trypsin/plasmin inhibitors (Mullins et al., 1980; Fazleabas et al., 1982) and will not be discussed further. Fraction IV, purple in

Fig. 2-1 Purification of uteroferrin from allantoic fluid of a day 67 pregnant pig. A, Sephadex G-100 chromatography. Allantoic fluid (approximately 4L) from a day 67 pregnant pig was dialyzed and then stirred with CM-cellulose at pH 8.2. Basic proteins were eluted with 0.5M NaCl, and then loaded onto a 5 x 90cm column of Sephadex G-100. Protein was monitored by absorbance at 280nm (A280). The peaks are labeled FIII, FIV and FV as explained in the text. B, CM-cellulose ion exchange chromatography. FIII proteins obtained by Sephadex G-100 chromatography (in A) were dialyzed and applied to a 1.5 x 10cm column of CM-cellulose. Elution was performed with a 250ml gradient (0.01-0.5M) of NaCl. Protein was monitored by absorbance at 280nm (A280) and purple coloration by absorbance at 545nm (A545). Uteroferrin eluted as a single peak between 0.20 and 0.25M NaCl.


A
F III
F IV
F V
A 280
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
80
FRACTION
B
A 280 (●)
2.0
1.5
1.0
0.5
0.0
0.5
0.25
NaCl (M)
0.5
0.3
0.1
A 545 (○)
15
30
45
60
FRACTION

color, contained uteroferrin. When FIV was pooled, dialyzed, loaded onto a column of CM-cellulose, and eluted with a linear sodium chloride gradient, uteroferrin eluted as a single symmetrical peak (Fig. 2-1B). Samples with an A_{280}/A_{545} ratio of less than 14.0 were pooled and considered to be pure uteroferrin (Buhi, 1982). In this typical preparation of uteroferrin from allantoic fluid, about 135mg of pure uteroferrin were obtained, which is considered to be an average yield (Roberts and Bazer, 1980). Fraction III had an obvious pink color and was demonstrated to be a high molecular weight form of uteroferrin, consisting of uteroferrin non-covalently associated with a second protein whose function is unknown (Baumbach et al., 1986). The yield of the high molecular weight uteroferrin, pooled immediately after the gel filtration step, was 47mg. The amount of FIII obtained varies greatly between animals, with uterine secretions from early pregnancy or pseudopregnancy containing more FIII than those from late pregnancy or pseudopregnancy (Baumbach et al., 1986). The specific activities of uteroferrin and high molecular weight uteroferrin from this preparation were shown to be approximately 230 units/mg protein and 106 units/mg protein, respectively. The total recoverable acid phosphatase activity from the allantoic fluid of this day 67 pregnant pig was greater than 3.6×10^4 units.

Purification of the Human Type 5 Acid Phosphatase from Hairy Cell Spleen

Levels of acid phosphatase in spleens from a patient with hairy cell leukemia and from a normal individual are presented in Table 2-1. Note that the spleen of the patient with hairy cell leukemia was greatly enlarged. In addition, the specific activity of the extract from the

TABLE 2-1

Tartrate insensitivity and activation by 2-mercaptoethanol of acid phosphatase activity in spleen homogenates from a patient with hairy cell leukemia and from an individual with a normal spleen

Patient	Weight of Spleen	Addition to Phosphatase Assay	Specific Activity
	kg		units/mg protein
1	2.3	None	0.453
		0.01M NaK tartrate	0.483
		0.1M 2-mercaptoethanol	0.662
		None	0.011
2	0.22	0.01M NaK tartrate	0.007
		0.1M 2-mercaptoethanol	0.012

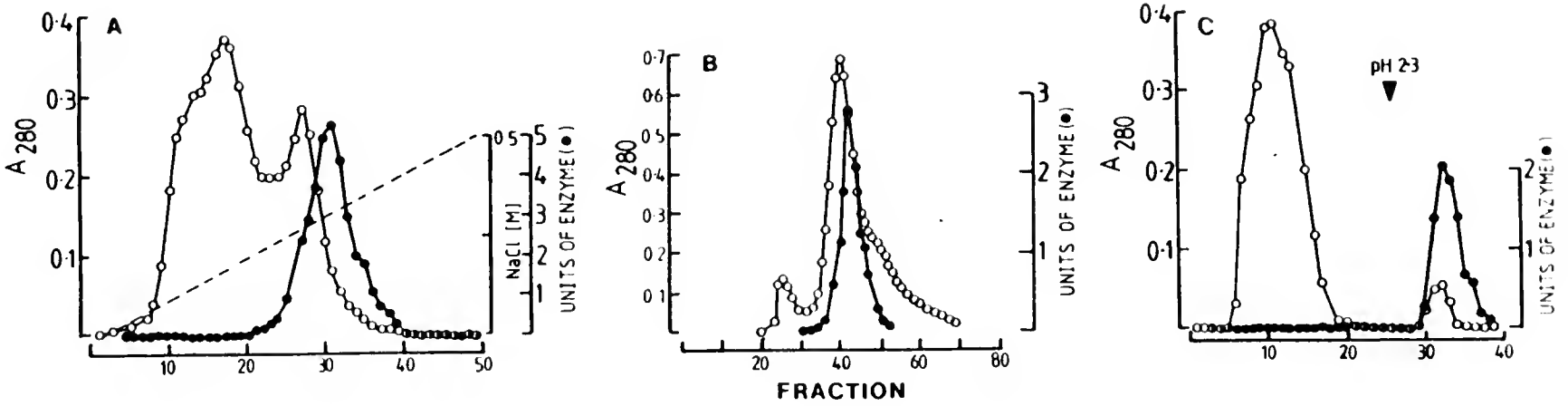
Results from spleen 1 (weight 2.3kg) were obtained from 30,000xg supernatant fraction of a slice (250g) of tissue that had been frozen (-80°C) for 2 weeks prior to assay. Results from spleen 2 were obtained from the 30,000xg supernatant fraction from freshly homogenized tissue of the entire organ which weighed 0.22kg. NaK tartrate is Rochelle's salt and corresponds with the L-(+)-form of tartrate.

large hairy cell spleen was about 40-fold higher than that from the normal spleen. Moreover, the acid phosphatase activity from this spleen was unaffected by the addition of 10mM tartrate but was stimulated by the presence of 0.1M 2-mecaptoethanol. By contrast, tartrate caused almost a 40% inhibition of the total acid phosphatase activity in the extract of normal spleen, and there was only a slight activation with 2-mercaptoethanol.

Two procedures have been employed for purification of the tartrate-resistant acid phosphatase in the extract from the hairy cell spleen. The first is illustrated in Fig. 2-2 and employed a gradient salt elution from a column of CM-cellulose (Fig.2-2A), gel filtration on Sephadex G-100 (Fig. 2-2B) and, as a final step, immunoaffinity chromatography (Fig. 2-2C). The acid phosphatase, which is very basic, was eluted from CM-cellulose at a salt concentration of 0.25M. Upon gel filtration the enzyme emerged as a peak of apparent M_r=34,000, about one fraction behind the elution position of highly purified uteroferrin. The fractions containing the enzyme were pooled and loaded directly onto an immunoaffinity column consisting of anti-uteroferrin antibodies covalently linked to Sepharose 4B. All of the phosphatase activity bound and could be quantitatively eluted using low pH buffer.

In the second procedure, the dialyzed extract of the spleen was applied batchwise to CM-cellulose at pH 8.2 and the acid phosphatase activity was eluted with 0.5M NaCl. Rather than subjecting the enzyme which had bound to further CM-cellulose ion exchange chromatography and gel filtration, the preparation was loaded directly onto the anti-uteroferrin immunoaffinity column. This abbreviated procedure provided

Fig. 2-2 Purification of human spleen phosphatase. A, CM-cellulose ion exchange chromatography. Post mitochondrial supernatant fraction was dialyzed and then stirred with CM-cellulose at pH 8.2. The enzyme was eluted with 0.5M NaCl; the resulting eluate was dialyzed and then reapplied to a 1.5 x 10cm column of CM-cellulose. Elution was performed with a 250ml gradient (0.01-0.5 M) of NaCl. Acid phosphatase activity eluted between 0.25 and 0.35M NaCl. Units of enzyme (●) are here represented by absorption values at 410nm obtained by incubation of 2.5 μl samples in 1ml of 0.1M acetate buffer, pH 5.3, containing 20mM p-nitrophenylphosphate at 37°C for 10 minutes. Protein (O) was monitored by absorbance at 280nm (A_{280}). B, Sephadex G-100 chromatography. Enzyme, partially purified by CM-cellulose ion exchange chromatography, was loaded onto a 1.5 x 80cm column of Sephadex G-100. Enzyme and protein were assayed as in A. C, immunoaffinity chromatography. Enzyme from the Sephadex column was loaded onto the anti-uteroferrin antibody affinity column. The phosphatase was eluted with glycine buffer, pH 2.3. The beginning of the elution is indicated by an **arrowhead**. Enzyme and protein were assayed as in A.


A
B
C
A280
NaCl (M)
UNITS OF ENZYME(•)
FRACTION
pH 2·3

a greater than 500-fold purification from the extract of the hairy cell spleen within 24 hours (Table 2-2). The final specific activities have varied between about 100 and 500 units/mg of protein.

Major losses of activity occurred whichever procedure was employed. Collection of the enzyme on CM-cellulose was particularly inefficient. However, if this step was repeated several more times (results not shown) the percent of recovery of enzyme from the original extract could be increased to over 40%. The phosphatase activity in the 30,000xg supernatant fraction from the spleen extract was relatively unstable. Storage at 4°C led to a complete loss of activity within 48 hours. Samples kept at -20°C lost over 60% of their activity in 20 days. The purified enzyme, on the other hand, appeared to relatively more stable when frozen at -20°C, and samples have been stored successfully for up to 2 months with retention of most of their original activity.

When the immunoaffinity-purified enzyme was analyzed by SDS-PAGE it gave a major protein band of apparent M_r=34,000 and two minor bands of about M_r=20,000 and 14,000 (Fig. 2-3). The relative amounts of the higher molecular weight species and the two lower molecular weight bands varied between preparations from the same spleen (results not shown).

The purified enzyme was also examined by electrophoresis under nondenaturing conditions (Fig. 2-4). The gels were then "stained" for acid phosphatase activity. The hairy cell enzyme (lane 3) gave two bands of activity, a minor fast migrating species and a dominant slower band. Uteroferrin also gave 2 bands, but the slower migrating band in this case was scarcely visible (lane 2). A commercial standard for

TABLE 2-2

Purification of tartrate-resistant acid phosphatase from spleen of a patient with hairy cell leukemia

Purification Step	Total Protein	Total Activity	Recovery	Specific Activity	Fold Purification
	mg	units	%	units/mg	X
30,000xg	5,060	3,230	100	0.64	
CM-cellulose	3.4	674	20.9	198	309
Affinity column	0.41	145	4.2	358	560

A 212g piece of a 2.4kg spleen was homogenized in lysis buffer. The homogenate was centrifuged at 30,000xg and the enzyme was purified from the supernatant fraction. Activities represent enzyme activated with 0.1M 2-mercaptoethanol at pH 5.3 for 10 minutes.

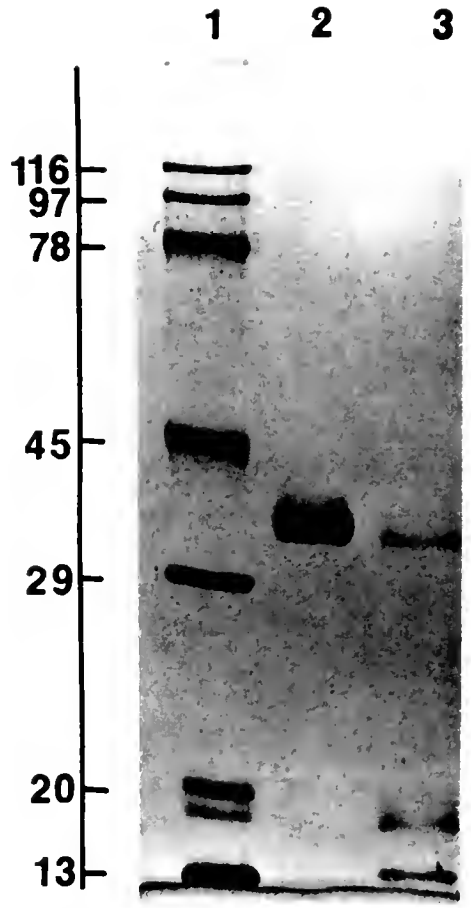


Fig. 2-3 SDS-polyacrylamide gel electrophoresis of purified uteroferrin and hairy cell phosphatase. The scale on the left is molecular weight x 10^{-3}. Lane 1, molecular weight standards; lane 2, purified uteroferrin; lane 3, hairy cell phosphatase following immunoaffinity chromatography. The gel was 10% (w/v) polyacrylamide; it was stained with Coomassie Blue. Note that the hairy cell enzyme gave major bands at 34,000, 20,000 and 14,000.

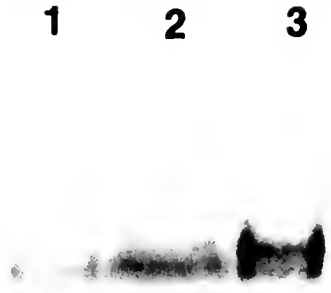


Fig. 2-4 Polyacrylamide gel electrophoresis of acid phosphatases at pH 5.4 in β-alanine buffer. Gels were stained with phosphatase substrate, α-naphthylphosphate, and hydrolysis product coupled with Fast Garnet GBC salt. Lane 1, Type 5 isozyme electrophoretic standard from Calbiochem-Behring; lane 2, uteroferrin; lane 3, hairy cell enzyme immunoaffinity purified from spleen. Note that a standard isozyme 5, uteroferrin, and the hairy cell phosphatase gave similar, but not identical, electrophoretic patterns. Migration (towards the cathode) was from the top to bottom.

human isozyme 5 is shown in lane 1. Its pattern resembled that of uteroferrin.

Purification of the Type 5 Isozyme from Normal Human Spleen

An isozyme with a high cathodal mobility has been detected in extracts of normal spleen by others (Lam et al., 1973). In addition, the extract of spleen described in Table 2-1 contained significant amounts of a tartrate-resistant acid phosphatase activity. Therefore an attempt was made to isolate the enzyme from the total extract of a normal spleen by means of procedure 2. Approximately 16 units of enzyme activity bound to CM-cellulose at pH 8.2. This represented less than 5% of the total acid phosphatase activity in the extract. A total of 6 units were recovered from the immunoaffinity column. This final preparation was insensitive to L-(+)-tartrate and was activatable by 2-mercaptoethanol. Upon electrophoresis in nondenaturing gels it gave two cathodally migrating bands of activity. These were identical to the bands of activity seen with the hairy cell enzyme (results not shown). The results confirm that the Type 5 phosphatase is present as a minor component of spleens of normal individuals.

Purification of the Type 5 Phosphatase from Human Placenta

Because of the difficulty in obtaining human spleens, it was necessary to determine whether other tissues could be used as a source of the human tartrate-resistant acid phosphatase. Human placenta can be obtained fresh, in large quantities, at any time. Therefore, this tissue was tested for the presence of the enzyme. Two placentas were studied, and a slightly different purification protocol was used for each one. A tartrate-resistant acid phosphatase was purified from each

placenta. Figure 2-5 shows that the placenta enzyme, like the spleen enzyme, has an apparent Mr of 34,000. Interestingly, the two minor bands found associated with the placenta enzyme are of different apparent Mr values than those associated with the spleen enzyme. Human placenta can contain about as much Type 5 phosphatase as normal human spleen (Table 2-3) when measured per gram of tissue. Note that human hairy cell spleen contains at least 20-fold more Type 5 isozyme per gram of tissue than the other sources of the human enzyme.

Purification of the Bovine Spleen, Bovine Uterine and Rat Spleen Phosphatases

Table 2-3 also shows the yields of the purple, iron-containing phosphatases from other sources. Davis et al. (1981) claim that they can purify up to 2mg of purple phosphatase per 2kg of beef spleen, but that the yield varied greatly between animals. The yields obtained in this study were consistently lower than 0.5mg/kg (data not shown). The yield of the bovine uterine enzyme was considerably less than that of the porcine enzyme on a per animal basis (Table 2-3). The yield of the rat spleen enzyme was quite high (Table 2-3) and comparable to that of Hara et al. (1984).

pH Optima of the Type 5 Phosphatases

The pH optima for the various Type 5 phosphatases are listed in Table 2-4. The values range from pH 4.2 for the bovine uterine enzyme to pH 6.0 for the bovine spleen enzyme.

Glycoprotein Nature of the Type 5 Phosphatases

Porcine uteroferrin is known to contain a single high-mannose oligosaccharide chain (Saunders et al., 1985). The rat spleen enzyme (Hara et al., 1984), and the bovine spleen enzyme (Davis et al., 1984)

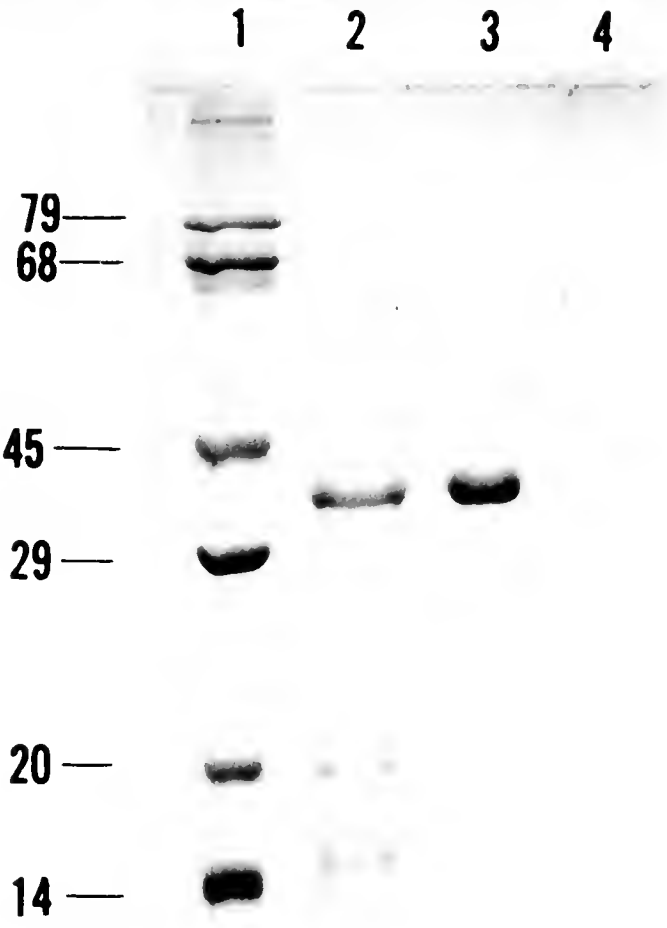


Fig. 2-5 SDS polyacrylamide gel electrophoresis of uteroferrin, the phosphatase from human spleen and the phosphatase from placenta. The molecular weight standards are shown in lane 1. Note that the spleen enzyme (lane 2) gave major bands at 34,000, 20,000 and 15,000, while the placenta (lane 4) gave major bands at 34,000, 16,000 and 13,000. Uteroferrin (lane 3) has an apparent Mr of 35,000. Approximately 20μg of protein were present in each lane. Gels were stained with Coomassie blue. The gel was composed of 12.5% (w/v) polyacrylamide. The scale on the left is molecular weight x 10^3.

TABLE 2-3

Levels of tartrate-resistant acid phosphatase acitivity in various tissues

Source of Enzyme	Units of Enzyme Activity	Units of Enzyme Activity per 100g Tissue
Human placenta 1 (630g)	19	3.0
Human placenta 2 (650g)	7	1.1
Normal human spleen (220g)	6	2.7
Hairy cell spleen (212g)[a]	145	68.3
Beef spleen (200g)[a]	4.5	4.5
Rat spleen (2g)[b]	0.74	37
Bovine uterine fluids (400ml)[c]	224	-
Porcine allantoic fluids (4L)[c]	36,400	-

[a]Approximately one-tenth the total tissue mass
[b]Obtained from three animals
[c]Obtained from one animal

Purified enzymes were incubated with 0.1M 2-mercaptoethanol and assayed at their pH optima with 20mM p-nitrophenylphosphate. One unit of enzyme hydrolyzes the release of 1μmol of p-nitrophenol per minute.

TABLE 2-4

pH optima for the tartrate-resistant acid phosphatases

Enzyme	pH Optimum
Porcine uteroferrin	4.9
Human hairy cell enzyme	5.3
Bovine spleen enzyme	6.0
Bovine uterine enzyme	4.2
Rat spleen enzyme[a]	5.0-5.8

[a] Hara et al., 1984

Enzymes were assayed with 0.1M sodium acetate, pH 3.0-5.4, 0.1M Tris acetate, pH 5.6-6.5, 0.1M Tris-HCl, pH 7.0-7.5, buffers in the presence of 0.1M 2-meracaptoethanol with 20mM p-nitrophenylphosphate as substrate.

were demonstrated to be glycoproteins. In this study it was demonstrated that the human spleen and placenta enzymes bind avidly to Con A-Sepharose. The enzymes could be eluted with 10mM α-methyl-mannoside at 50-60°C or with 0.1M acetic acid. These results indicate that the human Type 5 phosphatase, along with the purple, iron-containing phosphatases, contain high mannose or hybrid-type oligosaccharide chains.

Iron Content of the Type 5 Phosphatases

It has been demonstrated that the bovine spleen enzyme (Davis et al., 1981) and rat spleen enzyme (Hara et al., 1984) contain two atoms of iron per molecule of protein. However, Buhi et al. (1982b) consistently demonstrated that certain preparations of uteroferrin bound about 1 mol Fe/mol protein. Table 2-5 compares the iron contents of pink, high molecular weight uteroferrin, purple uteroferrin, the bovine uterine enzyme, the rat spleen enzyme, the bovine spleen enzyme and the human spleen enzyme. While pink, high molecular weight uteroferrin contains two atoms of iron per molecule of uteroferrin, purple uteroferrin preparations contain considerably less iron. Triplicate determinations on a single purified sample of the human enzyme revealed that it contained 2.2 $\pm$ 0.4 atoms of iron/molecule of protein.

Activation by Reducing Agents

Reports on the effect of reducing agents such as ascorbate and 2-mercaptoethanol on the activities of the Type 5 isozyme of human spleen have been at variance (Lam et at., 1977; Robinson and Glew, 1980). The degree of activation has been unpredictable throughout the course of this study. With partially purified enzyme

TABLE 2-5

The iron content of the tartrate-resistant acid phosphatases

Source of Enzyme	mmol Fe/mmol Protein
d65 allantoic fluid, purple porcine Uf	1.2[a]
d65 allantoic fluid, pink Mr=80,000 Uf	2.0[a]
d110 pseudopregnant fluid, purple porcine Uf	1.5[a]
d110 pseudopregnant fluid, pink Mr=80,000 Uf	2.3[a]
Human hairy cell spleen	2.2
Bovine spleen	2.1[b]
Bovine uterine fluid	1.4
Rat spleen	1.9-2.0[c]

[a]Baumbach et al., 1986
[b]Davis et al., 1981; Campbell et al., 1978
[c]Hara et al., 1984

The iron content was determined by the method of Cameron (1985) as modified by Campbell and Zerner (1973). Approximately 50-100μg of protein were used.

(post-CM-cellulose) a 2-3-fold activation was usually observed when enzyme was incubated with 0.1M 2-mercaptoethanol for 10 minutes at pH 5.3 before being assayed for activity. The degree of activation of crude enzyme (Table 2-2) was usually less than two-fold. However, some preparations of affinity-purified enzyme have shown negligible activity in the absence of reducing agent while other, more inherently active preparations were only stimulated about two-fold. The results with one purified preparation are summarized in Table 2-6. The extent of activation was clearly dependent upon the concentration of mercaptoethanol. Results with ascorbic acid (not shown) were similar.

Activation by mercaptoethanol leads to an increase in V rather than a change in K_m for both the human spleen enzyme and uteroferrin (Fig. 2-6A). Note that the K_m values for both enzymes at their pH optima (pH 5.3 and 4.9, respectively) are similar. Values for K_m between 0.75 and 3 mM have been reported for the spleen enzyme. V is also variable, the more active preparations giving values greater than 500 units/mg protein when pretreated with 2-mercaptoethanol.

Interestingly, pink, high molecular weight uteroferrin does not require pretreatment with 2-mercaptoethanol in order to exhibit maximal activity (data not shown).

Effects of Inhibitors on Phosphate Activity

The effects of a range of inhibitors on the human spleen enzyme and uteroferrin are very similar (Table 2-7). Both enzymes are completely inhibited by 0.1M dithionite, a reagent which is known to cause release of Fe from uteroferrin (Schlosnagle et al., 1976). Hydrogen peroxide and agents which interact with -SH groups also inhibit. The chelators

TABLE 2-6

Activation of purified human hairy cell phosphatase by 2-mercaptoethanol

2-Mercaptoethanol Concentration	Activity
M	% control
0	100
0.01	240
0.05	300
0.10	360
0.25	440

Enzyme was incubated with 2-mercaptoethanol for 20 minutes at pH 5.3 in 0.1M sodium acetate buffer prior to assay.

TABLE 2-7

Action of various potential inhibitors on the hairy cell acid phosphatase, uteroferrin, bovine spleen and bovine uterine acid phosphatases

Compound	Concentration	Acid Phosphatase			
		Human Hairy Cell	Porcine Uteroferrin	Bovine Spleen	Bovine Uterine
		% control			
Na dithionite	10^{-1} M	0	0	12	9
H_2O_2	0.01%(v/v)	75	73	37	67
Na iodacetate	10^{-3}M	72	85	ND[a]	ND
Na iodoacetamide	10^{-3}M	70	78	60	73
p-mercuribenzoate	10^{-4}M	42	57	ND	50
$FeCl_2$	10^{-5}M	45	58	ND	ND
NaEDTA	10^{-3}M	100	100	66	52
bipyridine	10^{-3}M	100	100	ND	ND
o-phenanthroline	10^{-3}M	ND	100	60	52
NaK tartrate	10^{-2}	100	100	95	100
NaK tartrate	10^{-3}M	100	100	ND	ND
Na phosphate	$5x10^{-3}$M	5	5	ND	ND
Na phosphate	10^{-3}M	40	37	43	28
Na phosphate	10^{-4}M	73	71	ND	ND
Na arsenate	10^{-3}M	18	8	ND	ND
Na arsenate	$5x10^{-4}$M	36	19	ND	ND
Na arsenate	$5x10^{-5}$M	83	74	67	29
Na molybdate	10^{-4}M	8	0	ND	ND
Na molybdate	10^{-5}M	4	12	ND	ND
Na molybdate	$5x10^{-7}$M	38	63	76	41
Na fluoride	10^{-3}M	60	67	53	ND
Na fluoride	$5x10^{-4}$	80	78	ND	ND

[a] ND, not determined

The enzymes were assayed in triplicate with p-nitrophenylphosphate as substrate (concentration 20mM). The enzymes were assayed at their pH optima (see Table 2-4). All activities are presented as per cent of a control assay performed simultaneously with no added inhibitor.

EDTA and bipyridine have no effect. Tartrate does not inhibit either phosphatase. Inhibition is observed with phosphate, arsenate, molybdate, and fluoride, however. Molybdate is the most effective, inhibiting strongly even at concentrations below 10^{-6}M; it appears to inhibit both enzymes in a noncompetitive manner (Fig. 2-6B).

Arsenate and phosphate are competitive inhibitors (Fig. 2-6C). Inhibition with 10^{-3}M fluoride (results not shown) is complex, and curvilinear Lineweaver-Burk plots are observed.

The effects of these inhibitors on pink, high molecular weight uteroferrin (data not shown) and the bovine uterine and spleen enzymes were quite similar to the results obtained for uteroferrin (Table 2-7).

Substrate Specificity for Phosphatase Activity

The substrate specificities of the human spleen enzyme and uteroferrin are quite similar (Table 2-8). p-Nitrophenylphosphate was the best substrate tested, but α-naphthylphosphate, pyrophosphate, and nucleotide tri- and di-phosphates are also hydrolyzed. Both enzymes hydrolyze ADP more effectively than ATP, whereas AMP is a poor substrate. Hexose phosphates are only very slowly hydrolyzed, if at all.

Both enzymes are phosphoprotein phosphatases and can release orthophosphate from the egg yolk protein phosvitin. The human spleen enzyme (1μg) had the ability to release 2.1nmol of orthophosphate from phosphvitin (10mg/ml) per minute at 37°C. The value for uteroferrin assayed identically was 1.5nmol of orthophosphate released per minute.

The substrate specificities of pink, high molecular weight uteroferrin (data not shown) and the bovine emzymes (Table 2-8) were

Fig. 2-6 Lineweaver-Burk plots of uteroferrin and hairy cell phosphatase activities in the presence of 2-mercaptoethanol and various inhibitors. A, activation of enzyme by 2-mercaptoethanol. In this experiment enzymes were activated with 0.1M 2-mercaptoethanol at pH 7.0 (uteroferrin) or 5.3 (hairy cell enzyme) for 10 min. Uteroferrin or hairy cell enzymes (0.15 μg) were assayed over a range of p-nitrophenylphosphate concentrations with the use of the standard assays with (O) and without (●) activation. B, effect of molybdate on acid phosphatase activity. Enzymes activated with 2-mercaptoethanol, were assayed as in A, either in the presence (●) or absence (O) of 10^{-7}M sodium molybdate. C, effect of arsenate and phosphate on acid phosphatase activity. Enzymes activated with 2-mercaptoethanol, were assayed as in A, except the amount of uteroferrin was less (0.05 μg/assay). Assays were carried out in the presence of either 5 x 10^{-4}M phosphate (Δ) or 5 x 10^{-5}M arsenate (●) or without inhibitors (O). Each point represents the average of three replicates. In A, B, and C assays with the hairy cell enzyme were carried out with purified enzyme that had been stored at -20°C for 3 weeks and which had lost about 50% of its initial activity. **Upper panels** represent results obtained with uteroferrin, **lower panels** with hairy cell phosphatase.

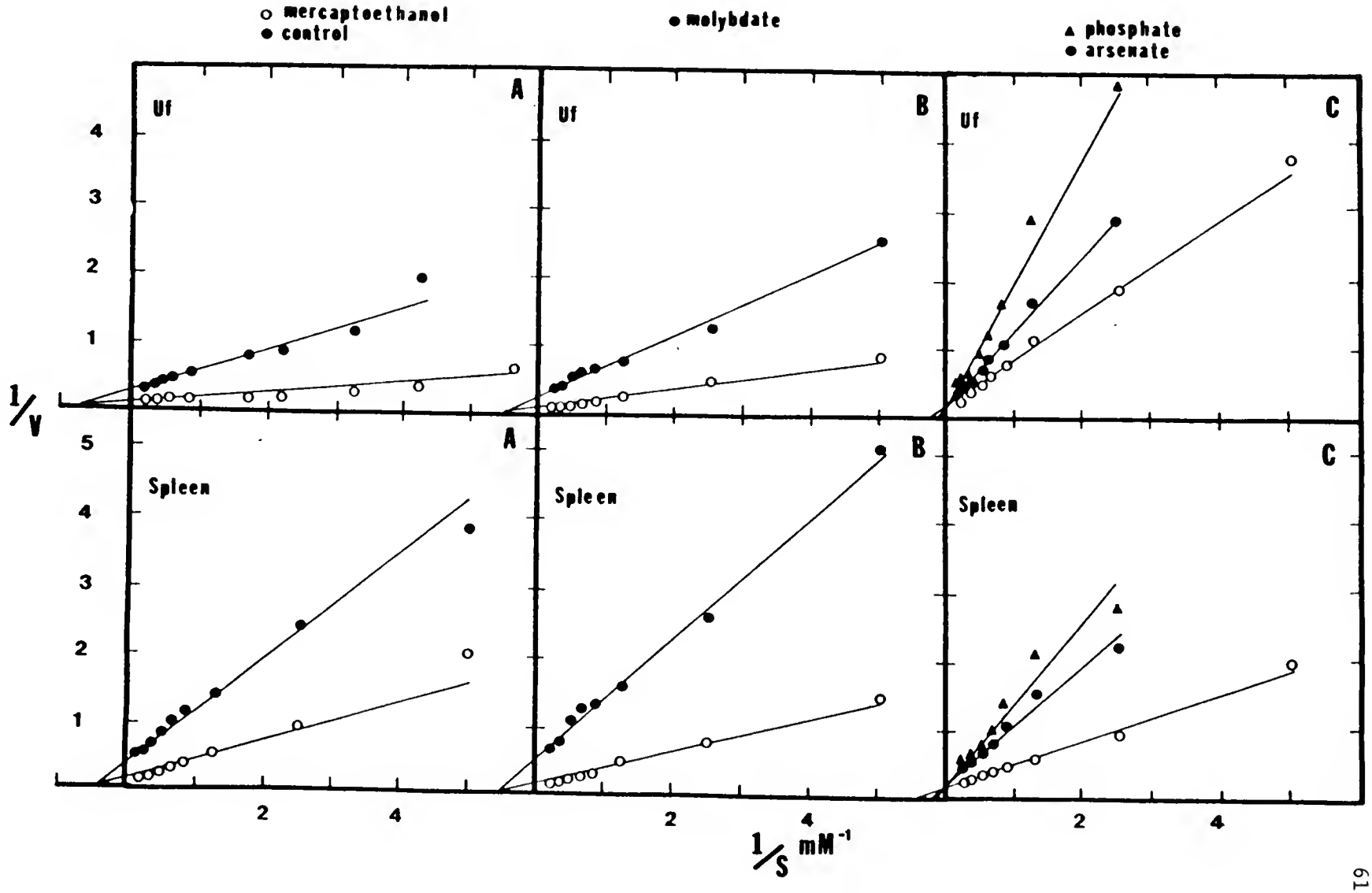

○ mercaptoethanol
● control
● molybdate
▲ phosphate
● arsenate
Uf
A
B
C
Spleen
1/V
1/S mM⁻¹

TABLE 2-8

Comparison of substrate specificities of hairy cell phosphatase, uteroferrin, bovine spleen and bovine uterine phosphatases

Substrate	Acid Phosphatase			
	Human Hairy Cell	Porcine Uteroferrin	Bovine Spleen	Bovine Uterine
		% control		
p-nitrophenylphosphate	100	100	100	100
α-naphthylphosphate	71	26	23	84
sodium pyrophosphate	24	52	7	45
ATP	49	36	20	3
ADP	92	81	18	87
AMP	4	2	3	10
D-glucose 6-phosphate	3	0		
D-mannose 6-phosphate	0	3		
D-fructose 6-phosphate	4	0		

All compounds were assayed at a concentration of 4mM at the pH optima of the enzymes.

generally similar to the results obtained with uteroferrin. However, the bovine uterine enzyme did not hydrolyze ATP well, and the bovine spleen enzyme was virtually inactive against pyrophosphate. The fact that each bovine enzyme was tested at its pH optimum (pH 6.0 for the spleen enzyme and pH 4.2 for the uterine enzyme) rather than at the same pH, may have influenced the results.

Immunological Cross-reactivity

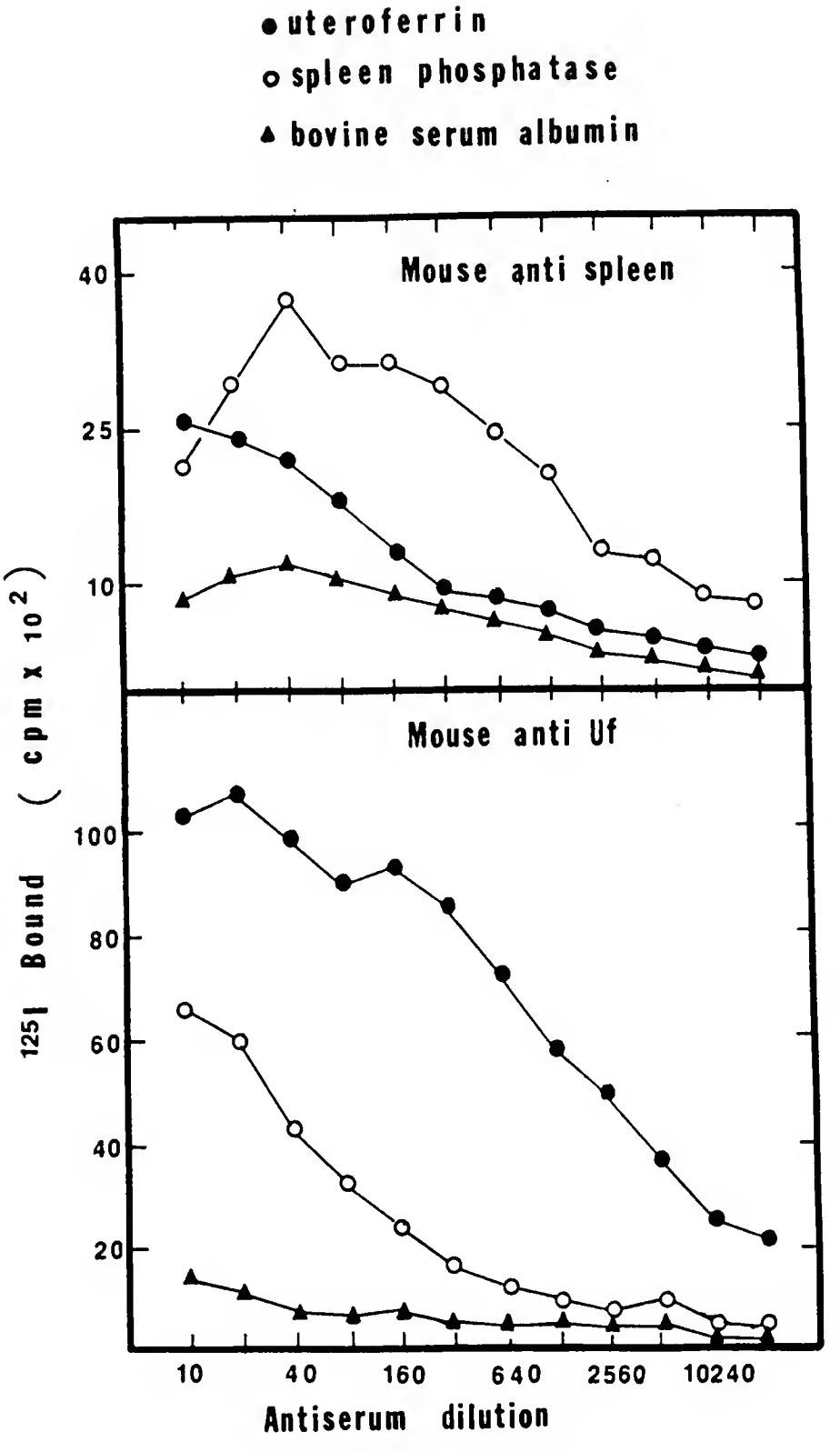
The human spleen enzyme clearly shows at least some immunological cross-reactivity with uteroferrin since it bound to anti-uteroferrin immunoglobulins immobilized on Sepharose (Fig. 2-2C). The extent of this cross-reactivity has been measured in a solid phase immunobinding assay which employed whole antiserum from mice, which had been immunized with either spleen enzyme (Fig. 2-7A) or uteroferrin (Fig. 2-7B). The antiserum to the spleen enzyme clearly bound to uteroferrin but binding was reduced by 50% when the antiserum was diluted 1:80. No binding could be detected below a 320-fold dilution. The titer towards the spleen enzyme was clearly much higher, with half-maximal binding occurring at an antiserum dilution of 1:1280.

When whole mouse antiserum raised against uteroferrin was employed, little antibody binding to the spleen enzyme was detectable at an antiserum dilution below 1:320. By contrast, binding to uteroferrin remained high down to an antibody dilution of 1:10,240. Therefore, the two proteins show only partial immunological cross-reactivity.

The Production of Monoclonal Antibodies Against Porcine Uteroferrin

The mice for fusion 5 were injected with high molecular weight pink uteroferrin. The results of this fusion, which was done in

Fig. 2-7 Solid phase radiobinding assay of whole mouse antiserum from mice immunized with human spleen phosphatase (**upper panel**) or uteroferrin (**lower panel**). Antibody was diluted serially and tested for binding to either uteroferrin (●), human spleen phosphatase (O), or bovine serum albumin (▲). Binding was measured by means of affinity purified ^{125}I-sheep anti-mouse IgG.


● uteroferrin
○ spleen phosphatase
▲ bovine serum albumin
Mouse anti spleen
Mouse anti Uf
125I Bound (cpm x 10^2)
40
25
10
100
80
60
40
20
10
40
160
640
2560
10240
Antiserum dilution

collaboration with George Baumbach, are outlined in detail in Baumbach (1984).

The mice for fusion 6 were injected with pure uteroferrin. Upon initial screening with uteroferrin as the antigen, 26 out of the 199 colonies screened were identified as positive. After each of these positive colonies was cloned by the limiting dilution method, 11 of the original colonies contained positive clones.

Three clones from fusion 5 and three from fusion 6 were chosen for further studies. These hybridomas, 5.58.1, 5.122.10, 5.127.3, 6.21.2, 6.22.1 and 6.37.4, were used to produce large quantities of antibody in mouse ascites fluid or culture media.

These antibodies were tested for binding to uteroferrin and pink, high molecular weight uteroferrin. All of the antibodies bound with high affinity to pink, high molecular weight uteroferrin. Only one antibody, 5.58.1, did not bind to uteroferrin. It appeared that 5.58.1 recognized the second, antigenically unrelated protein in FIII. This antibody will not be discussed further.

In order to determine which of these antibodies recognized the same epitope on the uteroferrin molecule (i.e., would compete with one another for binding to uteroferrin) and which recognized different epitopes (did not compete with one another for binding to porcine uteroferrin), competitive radiobinding assays were carried out.

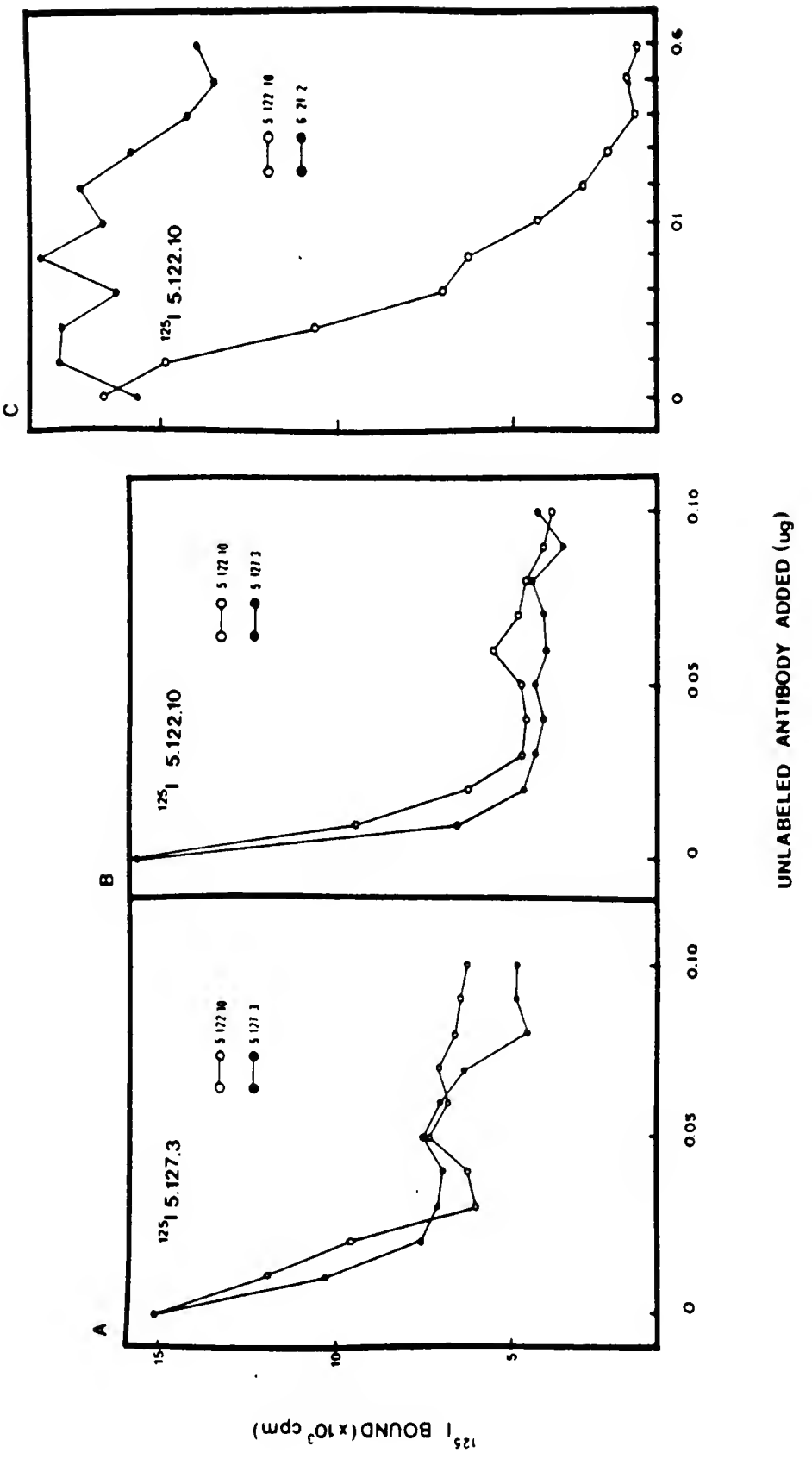
Each of the five monoclonal antibodies was iodinated to a specific activity of approximately 10^6 cpm/μg. Each iodinated monoclonal antibody was tested in a competitive binding assay with the same, unlabeled antibody. This was done to ensure that the binding abilities

of antibodies were not destroyed by iodination. Each labeled antibody was then tested with each remaining unlabeled antibody. The results of several of the competitive binding assays are shown in Fig. 2-8. Antibodies 5.122.10, 5.127.3 and 6.37.4 compete with one another for binding to porcine uteroferrin, even at very low concentrations of unlabelled antibody. Thus, these three monoclonal antibodies presumably interact with the same site on the uteroferrin molecule. Monoclonal antibodies 6.21.2 and 6.22.1 do not compete with each other, nor do they compete with 5.122.10, for binding to porcine uteroferrin. Each of these antibodies presumably recognizes a different site on the uteroferrin molecule.

The Production of Monoclonal Antibodies Against the Hairy Cell Spleen Enzyme

Hybridoma colonies derived from a mouse which had been immunized with the human spleen enzyme were screened for the production of antibodies which were able to bind both the spleen enzyme and to uteroferrin. From 200 wells containing live cells, 44 positive colonies were selected. When these positive colonies were rescreened, 29 remained positive. Only two of these colonies, 13.15 and 13.122, produced antibodies which bound as well or better to uteroferrin than to the human spleen enzyme. The dissociation constants for the binding of antibody 13.15 to the human enzyme and uteroferrin, respectively, were 22 and 16nM. The dissociation constants for the binding of antibody 13.122 to the human enzyme and uteroferrin, respectively, were 40 and 12nM. These two antibodies did not compete with one another for binding the porcine uteroferrin.

Fig. 2-8 Competitive binding of anti-uteroferrin monoclonal antibodies to uteroferrin adsorbed to flexvinyl microtiter wells. Uteroferrin (50 μg/ml; 0.05ml) was absorbed to the wells of flexvinyl plates. A sample of antibody 5.122.10 or 5.127.3 which had been iodinated to a specific activity of 10^6 cpm/μg was added to each well (0.05ml; 50,000 cpm). Samples of unlabeled antibodies were then added over a range of dilutions. The amount of ^{125}I which bound to the adsorbed uteroferrin was then measured. Results show that A, unlabeled 5.122.10 and 5.127.3 displaced the binding of radiolabeled 5.122.10; B, unlabeled 5.122.10 and 5.127.3 displaced the binding of radiolabeled 5.127.3; and C, 6.21.2 failed to compete with 5.122.10 for binding to uteroferrin.


A
B
C
^{125}I 5.127.3
^{125}I 5.122.10
^{125}I 5.122.10
^{125}I BOUND (x 10^3 cpm)
UNLABELED ANTIBODY ADDED (ug)

The Binding of the Monoclonal Antibodies to Other Tartrate-resistant Acid Phosphatases

In Fig. 2-9 the three monoclonal antibodies raised against porcine uteroferrin have been compared on the basis of their binding to uteroferrin, the human enzyme, and the purple, iron-containing rat spleen and bovine spleen acid phosphatases. All three antibodies bound to uteroferrin with very high affinity (Fig. 2-9 and Table 2-9) but bound relatively weakly to the beef spleen and human enzymes. The rat enzyme had an intermediate affinity (K_D=33nM) for antibody 6.21.2 and a relatively high affinity (8nM) for antibody 6.22.1. The binding of the anti-uteroferrin antibodies were also tested for binding to pink, high molecular weight uteroferrin and the bovine uterine enzyme. There is no difference in the binding of these monoclonal antibodies to uteroferrin and pink, high molecular weight uteroferrin (data not shown). The bovine uterine enzyme had a high affinity for antibody 5.122.10 (13nM) and low affinities for the remaining monoclonal antibodies. The bovine uterine enzyme clearly differs from the bovine spleen enzyme in its affinity for antibody 5.122.10 (13nM versus 120nM). Thus, the five phosphatases showed some immunological crossreactivities when tested in this manner, but the epitopes recognized by any one of the antibodies were clearly not identical.

Antibody 13.122, raised against the human spleen enzyme, was tested for its binding to the five phosphatases. Figure 2-9 and Table 2-9 show that this antibody had a slightly lower affinity towards the human enzyme (the antigen against which it had been raised) than towards uteroferrin or the rat spleen enzyme. The antibody bound as well to the bovine uterine enzyme as it did to the human enzyme, and bound most

Fig. 2-9 Binding of four monoclonal antibodies to uteroferrin and to the immunoaffinity purified phosphatases from human, bovine, and rat spleens. The enzymes (50 g/ml; 0.05ml) were allowed to adsorb to the wells of flexvinyl plates. Purified 5.121.10, 6.21.2, 6.22.1, which had been raised against uteroferrin, and 13.122, which had been raised against human spleen enzyme were diluted over a range of concentrations from about 10^{-6} to 10^{-10}M and added to the wells. Binding in this experiment was assessed by means of ^{125}I-labeled sheep anti-mouse IgG as described under "Materials and Methods." The graphs show the amount of antibody bound plotted against antibody concentration for the four enzymes. Uteroferrin, ●; human spleen phosphatase ▲ ; bovine spleen phosphatase 0; rat spleen phosphatase △ . The antibody used in each series of experiments (5.122.10, 6.21.2, 6.22.1, and 13.122) is shown on each graph.

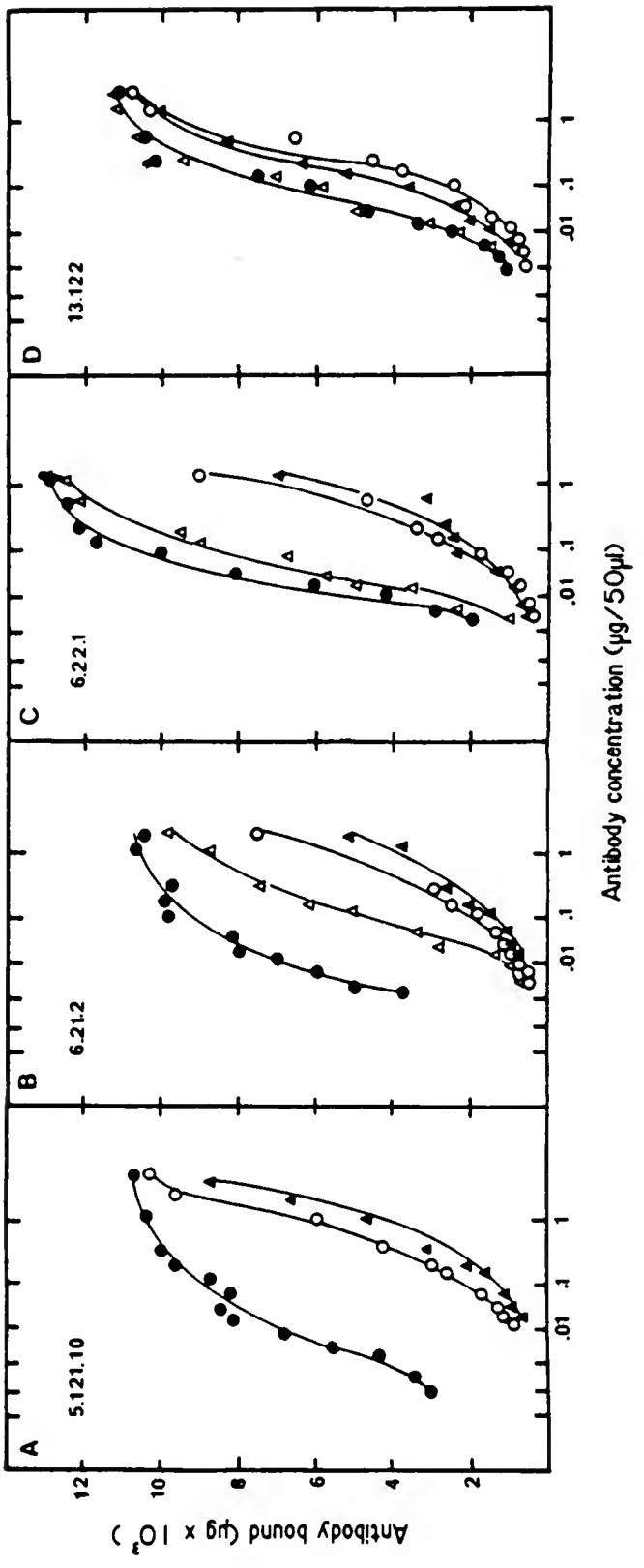

A
5.121.10
B
6.21.2
C
6.22.1
D
13.122
Antibody bound (µg x 10^3)
12
10
8
6
4
2
.01
.1
1
Antibody concentration (µg/50µl)

TABLE 2-9

Dissociation constants (K_D values) for binding of monoclonal antibodies to uteroferrin, the human spleen enzyme, the rat spleen enzyme, the bovine spleen and uterine enzymes

Antigen	Monoclonal Antibody			
	5.122.10	6.21.2	6.22.2	13.122
	K_D, nM			
Uteroferrin	1.0	1.2	2.7	12
Enzyme from hairy cell spleen	145	600	270	40
Enzyme from rat spleen		33	8	13
Enzyme from beef spleen	120	130	125	80
Enzyme from cow uterus	13	117	75	40

Monoclonal antibodies 5.122.10, 6.21.2 and 6.22.1 were produced by hybridoma clones which orginated from a mouse immunized with uteroferrin. They were selected by their ability to bind uteroferrin with high affinity in a solid phase radiobinding assay. Monoclonal antibody 13.122 was produced by a hybridoma clone which originated from a mouse immunized with the enzyme purified from human spleen. It was selected because of its ability to bind uteroferrin and the human spleen enzyme with approximately similar affinities.

weakly to the beef spleen enzyme (Table 2-9). However, the K_D values for all five phosphatases were not markedly different, a result which suggests that antibody 13.122 recognized a relatively conserved site.

Inhibition of Enzymatic Activity by the Binding of Monoclonal Antibodies

The acid phosphatase activity of uteroferrin was measured (without the addition of 2-mercaptoethanol) after each of the monoclonal antibodies had been allowed to bind to the enzyme at pH 7.0 for 1 hour. Table 2-10 demonstrates that antibody 5.127.3 effectively inhibited acid phosphatase activity when a 2-4-fold molar excess of antibody was added. Monoclonal antibody 6.22.1 inhibited the enzyme activity slightly at lower concentrations, while antibody 6.21.2 did not inhibit activity at all. Monoclonal antibody 13.122 inhibited the phosphatase activity about 30% at all concentrations tested. None of these antibodies were able to inhibit the human, rat or bovine spleen enzymes at the concentrations tested (data not shown).

Discussion

Tartrate-resistant acid phosphatases from various tissue sources can be purified by a procedure which closely resembles the fast, convenient purification protocol for porcine uteroferrin. This is particularly important in the case of the human enzyme, where previous attempts at purification to homogeneity were either not successful (Lam et al., 1977) or involved a large number of time-consuming steps (Robinson and Glew, 1980).

Three properties allowed the hairy cell phosphatase to be purified readily from extracts of spleen. The high isoelectric point ensured that it bound to CM-cellulose at high pH. Its immunological

TABLE 2-10

Inhibition of acid phosphatase activity of uteroferrin by monoclonal antibodies

Antibody	Molar Ratio, Antibody: Uteroferrin	Acid Phosphatase Activity
		% control
5.127.3	0.5:1	53
	2:1	22
	4:1	24
	8:1	42
6.21.2	0.5:1	97
	2:1	86
	4:1	109
	8:1	138
6.22.1	0.5:1	71
	2:1	68
	4:1	90
	8:1	111
13.122	0.5:1	79
	2:1	73
	4:1	73
	8:1	72

Uteroferrin (0.06mg) was incubated for 1 hour at room tempeature with the antibody specified, then assayed in triplicate for acid phosphatase activity with 20mM p-nitrophenylphosphate as substrate and without the addition of 2-mercaptoethanol. All activities are presented as percent of a control assay performed simultaneously.

cross-reactivity with anti-uteroferrin antibodies permitted it to be adsorbed to the immunoaffinity column. Finally, it was relatively stable to the conditions of elution, namely a brief exposure to pH 2.3 glycine buffer. It is conceivable that some of the losses that occurred at the immunoaffinity step did result from the elution conditions. Uteroferrin, although relatively stable to low pH, does begin to lose its phosphatase activity and presumably its bound iron if maintained below pH 3.0 (Campbell and Zerner, 1973). However, the major loss of yield occurred during the binding to CM-cellulose, a step which rarely provided more than 20% recovery of enzyme. This failure of the cation exchanger to bind all of the enzyme was not the result of its binding capacity being exceeded nor to the presence of enzyme variants of lower isoelectric point. Neither was it related to the presence of detergent. Possibly the enzyme remains complexed with some soluble, strongly anionic component in the homogenate. Whatever the basis of the phenomenon, repeated treatment with CM-cellulose does provide a much improved yield, with up to 40% of the total phosphatase activity of the spleen extract eventually binding.

When enzyme was purified from human placenta, protamine sulfate was added in order to bind various anionic components in the homogenate. This procedure was first employed by Glomset and Porath for purification of the bovine spleen enzyme (1960) and was also used by Anderson and Toverud in purification of the rat bone enzyme (1986). Protamine sulfate precipitation of anionic components is now routinely empolyed by our laboratory for the purification of tartrate-resistant acid phosphatases (see Allen et al., in press).

Despite the fact that the spleen was homogenized in a buffer which contained a wide range of protease inhibitors, there was evidence to suggest that much of the enzyme may have been cleaved into "subunits" during or prior to purification. Activity was lost, for example, if the homogenate was not rapidly processed, and the relative yield of 34,000 monomer and of the two subunits varied from preparation to preparation. Moreover, the final enzyme, although exhibiting a symmetrical peak of protein and enzyme activity of apparent M_r=34,000 during gel filtration on Sephadex G-100 always displayed some subunits during SDS-PAGE. Robinson and Glew (1981), working with the closely similar Gaucher spleen enzyme, have shown that the presence of the two lower molecular weight polypeptides could be minimized, but not prevented, if a protease inhibitor was included in the homogenizing buffer. In addition, when uteroferrin was treated with low concentrations of trypsin or chymotrypsin (Buhi, 1981) or incubated in allantoic fluid (Buhi et al., 1982b) an active enzyme with subunits of apparent M_r=20,000 and 15,000 was produced. Presumably both proteins possess a region of peptide which is sensitive to proteases. The reports that the closely similar beef spleen enzyme, which we have shown to crossreact immunologically with uteroferrin, possesses two such subunits (Davis et al., 1981; Campbell et al., 1978) implies that the bovine enzyme might also become cleaved proteolytically either during normal <u>in vitro</u> processing or during its isolation from the spleen homogenate. In contrast, the bovine bone enzyme (Lau et al., 1985, 1987) and the bovine uterine enzyme do not seem to be as sensitive to this proteolysis, they appear as intact monomers after purification. The human bone enzyme, unlike

the human spleen and placenta enzymes, is also easily purified without evidence of proteolytic products (Allen et al., in press).

The earlier report that the hairy cell enzyme is not activated by reducing agents (Lam et al., 1977) can probably be explained by the observation that activation was transient and varied between preparations. Similar observations have been made with uteroferrin. The differences in molecular weight (64,000 versus 34,000) can in turn by attributed to the fact that hemoglobin was employed as a standard in the earlier work (Lam et al., 1977) and assumed to maintain the molecular weight of the tetramer during sucrose density gradient centrifugation. The variability in kinetic parameters (particularly K_m and V) are probably best explained by the sensitivity of these enzymes to a variety of inhibitors, including sulfhydryl agents, anions, heavy metals, and oxidizing agents. The properties of the hairy cell enzyme do, however, differ somewhat from those reported for the tartrate-insensitive phosphatase isolated by Robinson and Glew (1980) from spleens of patients with Gaucher's disease. That phosphatase, despite the fact that it appeared to crossreact immunologically with the hairy cell enzyme (Lam et al., 1981), failed to utilize pyrophosphate as a substrate and hydrolyzed ATP considerably better than ADP (Robinson and Glew, 1980). It is still not clear, therefore, whether or not the Gaucher phosphatase and the one from hairy cell spleen are identical proteins.

The experiments with whole antiserum and with the different monoclonal antibodies to uteroferrin and the human spleen enzyme emphasize that the two proteins must have cross-reacting and presumably

structurally related determinants (epitopes) on their surfaces. Monoclonal antibody 13.122, for example, was selected for its ability to bind to both uteroferrin and to the spleen enzyme. Its binding characteristics towards both proteins and towards the rat spleen, bovine spleen and bovine uterine enzymes appeared fairly similar, suggesting that the epitope it recognized was relatively conserved. On the other hand, the monoclonal antibodies 5.122.10, 6.21.2 and 6.22.2 bound to the human, rat, and bovine spleen enzymes and the bovine uterine enzyme with much lower affinity than to uteroferrin. It can be concluded that all of the enzymes carry structurally homologous epitopes on their respective surface, but these sites are not equally conserved. Nevertheless, the ability to generate both polyclonal and monoclonal antibodies which have broad immunological crossreactivity should prove useful in further studies on the biosynthesis and localization of this class of acid phosphatase. These antibodies may have diagnostic value both for identifying leukemic hairy cells immunocytochemically and for measuring the level of the Type 5 isozyme in plasma by immunoassay. The latter procedure may be particularly useful for assessing osteoclast activity in individuals exhibiting abnormal bone metabolism (Minkin, 1982; Chen et al., 1975; Stepan et al., 1983) or in patients with osteoclastic tumors (Tavassoli et al., 1980).

The results of this study clearly demonstrate that uteroferrin, the purple-colored acid phosphatases from rat spleen, bovine spleen and the bovine uterus, and the enzyme from the spleens of human patients with hairy cell leukemia are closely related proteins. In addition to their immunological cross-reactivity, they are similar in kinetic properties,

in their substrate specificities, and in their sensitivities to a broad range of inhibitors. They also resemble each other in molecular size, isoelectric point, glycoprotein nature, and iron content. One obvious difference, however, between uterine enzymes and the spleen enzymes studied in this chapter is that the uterine enzymes are secreted glycoproteins whereas the others are principally intracellular. Nevertheless, even this paradox may be resolved as there is now considerable evidence that uteroferrin may represent a hypersecreted lysosomal enzyme (Baumbach et al., 1984). It is, for example, a substrate for the transferase which is specifically responsible for the selective phosphorylation of mannose residues on lysosomal enzymes (Lang et al., 1984). Uteroferrin is also secreted with the so-called lysosomal recognition marker, mannose 6-phosphate, present on its carbohydrate chain (Baumbach et al., 1984). Possibly all members of this class of acid phosphatase have the properties of lysosomal enzymes. Certainly many of them appear to reside intracellularly in inclusion bodies resembling lysosomes (Baumbach et al., 1984; Yam et al., 1971; Schindelmeiser et al., 1987).

The two bovine enzymes which were employed for comparative purposes in this study should perhaps be studied in more detail. Under the influence of progesterone, the bovine uterus secretes a uteroferrin-like protein which appears to be quite distinct from the bovine spleen enzyme, which is lysosomal. It has been demonstrated that the bovine uterine and spleen enzymes differ in a number of ways: pH optima (4.2 and 6.0, respectively), Mr (32,000 and 40,000), ability to hydrolyze pyrophosphate, and cross-reactivity with monoclonal antibody 5.122.10

(K_Ds 17 and 130nM). It would be interesting to determine whether differential targeting is due to variation in the enzymes' primary sequences. An alternative approach to study targeting of the purple phosphatases would involve the isolation and characterization of a porcine spleen enzyme which would presumably be lysosomal. It appears that such an enzyme exists, and is distinct from uteroferrin (M. Kazemi and R.M. Roberts, unpublished results).

In conclusion, it is proposed that the Type 5 human isozyme belongs to the growing class of iron-containing phosphatases of which uteroferrin and the phosphoprotein phosphatase of beef spleen are the best characterized. It remains to be determined whether the human enzyme possesses the purple color and characteristic g'=1.74 ESR iron signal which is exhibited by uteroferrin (Antanaitis et al., 1983) and the bovine spleen phosphatase (Davis and Averill, 1982). Why the Type 5 isozyme accumulates in the leukemic hairy cell and in the spleen histiocytes of patients with Gaucher's disease is unknown. Both these cell types are capable of phagocytosing erythrocytes (Robinson and Glew, 1980; Nanba et al., 1977), and the Gaucher cell is known to accumulate large amounts of iron (Robinson and Glew, 1980). Possibly the elevation of the Type 5 phosphatase is a reflection of abnormal iron metabolism in these cells.

CHAPTER 3
MOLECULAR CLONING OF THE TYPE 5, TARTRATE-RESISTANT ACID PHOSPHATASE FROM HUMAN PLACENTA AND ITS EXPRESSION IN LEUKEMIA CELLS

Introduction

There are at least six distinct types of acid phosphatase in human leukocytes which can be distinguished by electrophoretic and other characteristics (Li et al., 1979; Lam et al., 1973; Li et al., 1973). The Type 5 isozyme is the most cationic of the acid phosphatases and is the only isozyme insensitive to inhibition by L-(+)-tartrate. It has been detected in spleen, lung, liver and bone as a minor isozyme (Lam et al., 1973; Yam et al., 1971). However, it can become the dominant isozyme in certain pathological states. High tartrate-resistant acid phosphatase levels are often found within the spleen (Robinson and Glew, 1980) and monocytes (Troy et al., 1985) of patients with Gaucher's disease; the splenocytes and circulating white cells of patients with hairy cell leukemia (Yam et al., 1971); the spleens of patients with Hodgkin's disease (Drexler et al., 1986); and the sera of individuals undergoing active bone turnover (Yam, 1974). Elevated levels of this isozyme are also associated with various B-cell and T-cell leukemias (Drexler et al., 1986). In systematic studies of leukemia cell lines, it appeared that the tartrate-resistant, Type 5 acid phosphatase was not generally expressed in immature lymphoid cells, but rather by cells arrested in later stages of differentiation (Drexler et al., 1985, 1987a).

In the previous chapter the purification and characterization of this Type 5 acid phosphatase from human hairy cell spleen, normal spleen and placenta were reported. It was demonstrated that this human enzyme is remarkably similar to an abundant tartrate-resistant acid phosphatase secreted by the porcine uterus under the influence of progesterone, known as uteroferrin. Both the human enzyme and uteroferrin are iron-containing glycoproteins, and resemble each other closely in electrophoretic mobility, substrate specificity and sensitivity to a variety of activators and inhibitors. The two proteins are also immunologically related. While uteroferrin is believed to function in iron metabolism (see Roberts and Bazer, 1985; 1988), the function of the human enzyme is unknown.

In view of the clinical significance of the human tartrate-resistant acid phosphatase, further studies on its transcriptional control seemed warranted. A human placenta cDNA library was screened with polyclonal antibodies against porcine uteroferrin. However, these antibodies failed to detect a cDNA coding for the human tartrate-resistant acid phosphatase. Accordingly, two short cDNA clones coding for porcine uteroferrin were employed to isolate a cDNA coding for the human enzyme. In this chapter, the molecular cloning of a 1412 bp cDNA which covers the complete coding region of the human tartrate-resistant acid phosphatase is described, and the deduced amino acid sequence of the human enzyme is compared to the amino acid sequences of porcine uteroferrin and a related bovine spleen acid phosphatase. The expression of this human phosphatase in human leukemia cells is

demonstrated, and its regulation studied in the human erythroleukemia cell line K562.

Materials and Methods

Materials

The human placenta cDNA library, mouse spleen cDNA library and human placenta poly $(A)^+$ RNA were obtained from Clontech. Goat anti-rabbit and goat anti-mouse antibodies coupled to horseradish peroxidase were from Bio-Rad. All restriction endonucleases, the large fragment of E. coli DNA polymerase, T4 DNA ligase, and the RNA ladder were from Bethesda Research Laboratories. The [α-^{32}P]-dATP was purchased from New England Nuclear Corp. The 2'-deoxynucleotide triphosphate/2'3'-dideoxynucleotide triphosphate sequencing mixes were from New England BioLabs. Heparin-Agarose, hemin and TPA were from Sigma. Fetal bovine serum and RPMI 1640 were obtained from Gibco-BRL. Oligodeoxyhexamer for random primer extension probes was from Pharmacia. All other chemicals, reagent grade or better, were from Sigma or Fisher.

Methods

Preparation of antisera for immunoscreening. Adult New Zealand White rabbits were immunized with 0.2 to 0.5mg of uteroferrin mixed with Freund's complete adjuvant and then injected subcutaneously at multiple sites (Herbert, 1973). Booster injections of 0.2 to 0.5mg of antigen in incomplete adjuvant were administered monthly. Bleedings of 20 to 40ml were taken 10 days after injections. The samples were allowed to clot overnight at 4°C and then centrifuged to separate the serum from the clot. In some cases the antisera were affinity purified on anti-uteroferrin Sepharose as outlined in Chapter 2. In other cases the

antisera were run through a column of E. coli proteins which had been coupled to Sepharose, in order to remove antibodies against E. coli proteins (Huynh et al., 1984).

Screening of the lambda gtll libraries with anti-uteroferrin antibodies. A human placenta cDNA library prepared by Millan (1985) was purchased from Clontech. This library contains 1 x 10^6 independent clones, with 96% recombinant phage. The insert sizes range from 0.8-3.6 kb, and the average insert size is 1.8 kb. A mouse spleen cDNA library was purchased from Clontech. This library contains 1 x 10^6 independent clones, with 93% recombinant phage. The average insert size is 1.0 kb. The porcine uterine endometrium cDNA library was prepared by George Baumbach and Patrick Gillevet at the University of Florida. The method used for screening was developed by Young and Davis (1983a,b) and Huynh et al. (1984) and modified by deWet et al. (1984).

E. coli strain Y1090 was grown overnight in LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] plus 0.2% (w/v) maltose and 50μg/ml ampicillin at 37°C with good aeration. The next morning, the bacteria were centrifuged at 1500xg at room temperature for 10 minutes, and resuspended in one-fifth original volume of SM buffer [0.01M Tris-HCl, pH 7.5, 0.01M $MgCl_2$, 0.1mM EDTA, 0.2% (w/v) gelatin]. An appropriate number of phage were mixed with either 75μl of bacteria (for 100mm dishes) or 300μl bacteria (for 150mm dishes). The bacteria and phage were incubated at 37°C for 15 minutes to allow the phage to adhere to the bacteria. The phage and bacteria were then mixed with either 3 ml (for 100mm dishes) or 7.5ml (for 150mm dishes) of melted LB containing 0.7% (w/v) agarose, which had been kept at 45°C. The mixture

was poured onto plates containing LB with 1.5% (w/v) agar, which had been pre-warmed to 37°C. The plates were then placed in an incubator at 42°C for 3 to 4 hours, or until plaques began to appear on a confluent lawn of bacteria. Nitrocellulose filters, which had been soaking in 10mM isopropyl β-D-thiogalactopyranoside (IPTG) were then placed on the plates. The plates were incubated at 37°C for 4 hours which allowed a β-galactosidase fusion protein to be formed, which adhered to the filter.

After the filters had been removed, the plates were saved at 4°C until needed. The filters were washed three times with Tris buffered saline (TBS; 0.1M Tris-HCl, pH 8.0, 0.15M NaCl) and placed in antibody solution containing a 1:100 dilution of antisera in TBS with 3% (w/v) bovine serum albumin (BSA). The filters were incubated in antibody solution overnight at 4°C on a rocking platform at low speed.

The next day, the antibody solution was removed, filter sterilized through a Millipore 0.2μm filter, and stored at 4°C. (Each antibody preparation was used up to five times.) The filters were washed thoroughly with three changes of TBS. The additional protein binding sites on the nitrocellulose were blocked by incubation in TBS plus 3% (w/v) BSA. The filters were then treated with goat anti-rabbit antibody coupled to horseradish peroxidase diluted in TBS plus 3% (w/v) BSA at dilutions 3- to 5-fold more concentrated than the manufacturer's suggested dilution for Western blotting. The filters were incubated in the second antibody solution for 2 hours at room temperature on a rocking platform at low speed. The antibody was then removed and discarded, and the filters washed thoroughly as before.

The substrate solution used to detect immunoreactive fusion proteins colorimetrically was 0.33 mg/ml 4-chloro-1-naphthol in TBS plus 0.015% (v/v) hydrogen peroxide. When this solution was poured onto the washed filters, immunoreactive fusion proteins appeared as bright purple rings in less than 30 minutes.

Each time plaques were immunoscreened, dot blot positive controls were also tested. Dots of 1μl containing 1μg, 100ng, 10ng, 1ng and 0.1ng of uteroferrin were placed on a strip of nitrocellulose. These dot blots were treated exactly as the nitrocellulose filter lifts from plates. A good primary antibody with fresh secondary antibody could routinely detect 0.1ng of uteroferrin.

Plaques producing immunoreactive fusion proteins were removed as agarose plugs from the plates with sterile Pasteur pipettes or microcapillary tubes. Each plug was placed in 1ml of SM plus 50μl of chloroform and stored at 4°C. An appropriate volume of the phage in SM was rescreened as described as above. (An individual plaque typically contains 10^6-10^7 phage.) This process was continued until all plaques on a plate were positive.

General methods-handling DNA and RNA. DNA and RNA concentrations were estimated at 260nm assuming (E) 1%=250 and 200, respectively.

Ethanol precipitation-DNA was precipitated at -70°C for at least 15 minutes in the presence of 2.5M ammonium acetate, pH 7.5 and three volumes of 100% ethanol. Preipitates were collected by centrifugation (14,000xg; 15 minutes). RNA was precipitated for at least 1 hour at -20°C in the presence of 0.2M sodium acetate, pH 5.2 and two volumes of

100% ethanol. Precipitates were collected by centrifugation (14,000xg; 15 minutes).

Phenol/chloroform extration refers to the phase separation of a nucleic acid solution by emulsification with 1 volume of phenol, followed by re-emulsification in 0.5 volume of phenol and 0.5 volume of chloroform: isoamyl alcohol (24:1), and finally 1 volume of chloroform: isoamyl alcohol (24:1). The aqueous phase was collected each time by centrifugation (14,000xg; 15 minutes).

All solutions and materials that came in contact with RNA were autoclaved (when appropriate). Prior to autoclaving, the solutions were pretreated for 1 hour with a solution of 0.01% (v/v) diethylpyrocarbonate to inhibit RNases.

Screening of lambda gtll libraries with cDNA probes. The human placenta cDNA library from Clontech (see above) was screened with two cDNA probes, 13.1 and 4a3, which code for amino acids 157-186 and 218-260 of uteroferrin, respectively. The isolation and sequence analysis of these short clones have been described elsewhere (Simmen et al., 1988). The sequences of these cDNAs, and the inferred amino acid sequences, are shown in Fig. 3-1.

The screening method employed was that of Maniatis et al. (1982). An appropriate number of phage were plated as described above. The plates were incubated overnight at 37°C. The next day, nitrocellulose filters were placed on the plate, left for 1-3 minutes, then carefully removed. The DNA which had bound to the filters was denatured in solution of 0.5M NaOH and 1.5M NaCl. The filters were neutralized in 0.5M Tris-HCl, pH 8.0 containing 1.5M NaCl, followed by a wash in 1X SSC

Fig. 3-1 The nucleotide sequences of the cDNA clones coding for porcine uteroferrin used for screening the human placenta cDNA library and their deduced amino acid sequences. Numbers refer to the nucleotide position in the cDNA (top) or amino acid position in mature uteroferrin (bottom).

Uf Clone 13.1

1	CTG	GCG	CTG	GCC	CGC	AGA	CAG	CTG	GCC	TGG	ATC	AAG	AAG	CAG
157	L	A	L	A	R	T	Q	L	A	W	I	K	K	Q
43	CTG	GCG	GCA	GCA	AAG	GAG	GAC	TAT	GTG	CTG	GTG	GCC	GGC	CAC
171	L	A	A	A	K	E	D	Y	V	L	V	A	G	H
85	TAT	CCT												
185	Y	P												

Uf Clone 4a3

1	GGC	CAC	GAC	CAC	AAC	CTG	CAG	TAC	CTT	CAG	GAT	GAG	AAT	GGC
218	G	H	D	H	W	L	Q	Y	L	Q	D	E	N	G
43	TTG	GGC	TTT	GTG	CTG	AGC	GGG	GCC	GGG	AAC	TTC	ATG	GAC	CCC
232	L	G	F	V	L	S	G	A	G	N	F	M	D	P
85	TCC	AAG	AAG	CAC	CTG	CGC	AAG	GTC	CCC	AAC	GGC	TAC		
246	S	K	K	H	L	R	K	V	P	N	G	T		

(0.15M NaCl, 0.015M sodium citrate, pH 7.0). The DNA was baked onto the filters in a vacuum oven at 80°C for 2 hours. The filters were washed at 60°C in a solution of 0.05M Tris-HCl, pH 8.0 with 1M NaCl, 1mM EDTA and 0.1% (w/v) SDS for 30 minutes. Following the washings, prehybridization was carried out at 60°C in a solution containing 0.05M Tris-HCl, pH 7.5, 1M NaCl, 10mM EDTA, 0.1% (w/v) SDS, 0.2% (w/v) polyvinylpyrrolione, 0.2% (w/v) Ficoll type 400, 0.2% (w/v) BSA and 100μg/ml denatured, sheared salmon sperm DNA. The cDNA probes, labeled by the random primer extension method (see below), were allowed to hybridize to the DNA on the filters overnight at 60°C in the prehybridization buffer. Following hybridization, the filters were washed at 60°C with 1X SSC which contained 0.1% (w/v) SDS. Autoradiography was carried out at -70°C with DuPont Cronex Lighting Plus intensifying screens.

Generation of random primer extension probes. The ^{32}P-labeled cDNA probes used for screening the library were generated by the random primer extension method (Feinberg and Vogelstein, 1983). Isolated cDNA inserts (20-100ng), purified as described below, were boiled for 3 minutes to denature the DNA. The DNA was allowed to anneal to 25-50μg of a randomly generated oligodeoxyhexamer. The cDNA and primer were resuspended in a buffer containing 0.2M Hepes, pH 6.60, 5mM $MgCl_2$, 0.01M 2-mercaptoethanol, 0.05M Tris-HCl, pH 8.0, and 40μg/ml BSA. The deoxynucleotides dCTP, dTTP and dGTP were added at a concentration of 20μM, followed by the addition of 50-100μCi of [^{32}P]-dATP (800 Ci/mmol). The reaction was allowed to proceed overnight at room temperature. The following day, the reaction mixture was loaded onto a column

(1.5 x 25cm) of Sephadex G-50 in a buffer containing 0.05M Tris-HCl, pH 8.0 and 0.2M NaCl in order to separate unincorporated nucleotide from the radiolabeled probe.

Preparation of DNA from positive lambda gtll clones. The plate lysate method of Fritsch was employed (Maniatis et al., 1982) for isolation of phage DNA. Approximately 10^5 plaque forming units of a plaque pure positive phage were plated with E coli strain Y1088 and 0.8% (w/v) top agarose on each 150mm Petri dish containing NZYCM [1% (w/v) NZ-amine, 0.1% (w/v) casamino acids, 0.5% (w/v) yeast extract, 0.5% (w/v)NaCl, 0.2% $MgCl_2$, pH 7.5] with 1.5% (w/v) agarose. The plates were incubated at 37°C overnight. Lysis of greater than 90% of the bacteria was evident the next day. The plates were incubated at room temperature for 2 hours with 10ml SM in order to elute the phage from the agarose. The SM buffer was removed and the solution centrifuged at 10,000xg for 20 minutes to remove debris. The supernatant fraction, containing viable phage in protein coats, was incubated with 1μg/ml DNase I and 1μg/ml RNase A at 37°C for 1 hour. An equal volume of a solution of 20% (w/v) polyethylene glycol and 2M NaCl was added to the sample and left on ice for 1 hour. Precipitated phage were collected by centrifugation at 10,000xg for 30 minutes at 4°C.

The phage pellet was resuspended in 0.01M Tris-HCl, pH 8.0 and 1mM EDTA (TE) and SDS added to 1% (w/v) and EDTA to 10mM. The sample was incubated at 68°C for 15 minutes, then phenol/chloroform extracted (described above). The phage DNA was precipitated with isopropanol (described above). The DNA was resuspended in TE and stored at 4°C or

-20°C. The cDNA inserts were excised from lambda gtll by digestion with EcoR I in a buffer of 0.05M Tris-HCl, pH 8.0, 0.01M $MgCl_2$ and 0.1M NaCl. After electrophoresis on a 1% (w/v) agarose gel, the cDNA inserts were electroeluted from the gel.

Subcloning of cDNA inserts into pUC 19. The procedure employed was that of Hanahan (1983). A 3-fold molar excess of cDNA was added to 100ng of pUC 19 which had been previously digested with EcoR I. The cDNA were inserted into the EcoR I site and ligated for 2 hours at room temperature in a buffer containing 0.05M Tris-HCl, pH 7.8, 0.01M $MgCl_2$, 0.02M dithiothreitol, 1mM ATP, 1mg/ml BSA and 1 unit of T4 DNA ligase. One microliter of the ligation mixture was incubated with 100μl competent E.coli JM 83 (see below). After a 45 minute incubation on ice, and a subsequent 3 minute incubation at 42°C, the samples were spread on a plate of YT [0.8% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] containing 1.5% (w/v) agarose with 50μg/ml ampicillin, 0.025% (w/v) IPTG and 0.025% (w/v) 5-bromo-4-chloro-3indolyl β-D-galacto-pyranoside (x-gal). The plates were incubated overnight at 37°C. Only bacteria which contained plasmid grew on the plates because ampicillin was included; and colonies with cDNA inserts were white, those without inserts, blue.

Competent cells for transformation were prepared as follows: A log phase culture of E. coli strain JM83 was centrifuged at 1500xg for 10 minutes. The bacteria were resuspended in 1/5 volume of ice cold 0.05M $CaCl_2$ and incubated on ice for 45 minutes. The cells were centrifuged as before, and resuspended in 1/10 volume of ice cold 0.05M $CaCl_2$. The cells were aged at least one hour before being used for transformations.

Isolation of pUC 19 recombinant plasmids. The method of Ish-Horowicz and Burke (1981) was employed for isolating plasmid of interest from 5ml, 50ml and 1L cultures of E.coli JM83 containing that plasmid. The bacteria were pelleted at 1500xg for 10-30 minutes. The bacteria were resuspended in a solution of 0.05M glucose, 0.025M Tris-HCl, pH 8.0 and 0.01M EDTA. After a 10 minute incubation at room temperature, a solution of 0.2M NaOH and 1% (w/v) SDS was added to the bacteria. The samples were allowed to sit on ice for 10 minutes. A solution of 5M potassium acetate, pH 4.8 was added, and the samples centrifuged at 15,000xg for 30 minutes. The supernatant fraction, which contained the plasmid, was treated with 1μg/ml RNase A for 1 hour at 37°C. The samples were extracted with phenol/chloroform and the DNA ethanol precipitated.

Southern blots of DNA. Samples of DNA were subjected to electrophoresis on 1% (w/v) agarose gels as described above. The gel was treated with 0.2M HCl followed by 0.5M NaOH, then equilibrated in 0.05M Tris-Borate buffer, pH 8.0 with 1mM EDTA (TBE). The DNA was transferred to Gene Screen membrane by the method of Southern (1972). After a 16 hour transfer, the DNA was cross-linked to the membrane with UV light (Church and Gilbert, 1984).

Generation of oligonucleotide probes. Two redundant oligonucleotides which code for amino acids 11-16 and 241-245 of porcine uteroferrin were synthesized (see Fig. 3-2). Approximately 100pmol of oligonucleotide were suspended in a buffer of 0.1M Tris-HCl, pH 7.6, 0.05M $MgCl_2$, 5mM dithiothreitol, 1mM spermidine and 0.2mM EDTA. Ten units of T4 polynucleotide kinase and 200pmol [γ-^{32}P]-ATP (1.4mCi, 7000

Fig. 3-2 The sequences of the redundant oligonucleotides used for Southern blot analysis and their deduced amino acid sequences. Oligonucleotides coding for amino acids 11-16 of porcine uteroferrin and the beef spleen enzyme (KM28) and amino acids 240-245 of the same proteins (KM29) were synthesized at the University of Missouri DNA synthesis facility and purified by ethanol precipitation. Probe KM28 is 128-fold redundant, with an average GC content of 65% and a predicted Tm of 70°C; probe KM29 is 32-fold redundant, with an average GC content of 40% and a predicted Tm of 60°C. The sequence of human fibronectin was obtained from Kornblihtt et al. (1985).

```
                  11                                                  16
Uteroferrin:      Ala-Val-Gly-Asp-Trp-Gly

                                        T
Probe KM28:       GCN  GTN  GGN  GAC  TGG   GG
```

```
                  240                                           245
Uteroferrin:      Gly-Asn-Phe-Met-Asp-Pro

                       T    T         T
Probe KM29:       GGN  AAC  TTC  ATG  GAC   CC
```

```
                                      T
Probe KM28:       GCNGTNGGNGACTGGGG

Fibronectin:      GCCGUUGGAGAUGAGUG
(3660-3676)
```

Ci/mmol) were added and the reaction allowed to proceed at 37°C for 1 hour. Unincorporated [γ-^{32}P]-ATP was removed by ethanol precipitation. Probe KM28 had a Tm of 70°C and probe KM29 had a Tm of 60°C. Hybridization was carried out at Tm-25°C. The radiolabeled oligonucleotides were employed as probes for Southern blots of clone HP 6.1.

The Southern blots were prehybridized and hybridized in sodium phosphate buffer, pH 7.2 containing 7% (w/v) SDS, 1mM EDTA and 1% (w/v) BSA, at the appropriate temperatures. The blots were washed in 0.076M sodium phosphate buffer, pH 7.2 with 1% (w/v) SDS and 1mM EDTA, at the appropriate temperatures (Tm-25°C).

Cloning of cDNA inserts into M13. Competent E. coli strain TG-1 was prepared as described earlier for JM83. The cDNA inserts from positive clones were cloned into the EcoR I site of M13mp18 (Messing, 1983; Messing et al., 1977) as described earlier for pUC 19. Restriction fragments of the cDNA inserts were prepared as follows: The cDNA inserts were digested with Alu I, Msp I or Rsa I in a buffer containing 0.05M Tris HCl, pH 8.0 and 0.01M $MgCl_2$ with 10 units of enzyme/mg DNA; when restricted with Hae III, the buffer used was 0.05M Tris-HCl, pH 8.0, 0.01M $MgCl_2$ and 0.05M NaCl. In some cases the entire assortment of restriction fragments from each individual digest was cloned into the Sma I site of M13mp19, a procedure known as "shotgun" cloning. In other cases, the restriction fragments were separated on a 6% (w/v) polyacrylamide gel (Maniatis et al., 1982), and the restriction fragments 100 bp or larger cut out of the gel and electroeluted. Each fragment was then cloned into the Sma I site of M13mp18 individually.

One microliter of the ligation mix was incubated with competent E.coli TG-1 as described earlier for cloning into pUC19. The bacteria and phage were added to a mixture YT with 0.7% (w/v) agarose at 45°C, containing 0.025%(w/v) x-gal, 0.025% (w/v) IPTG and 100μl log phase E. coli TG-1. The mixture was poured onto a 100mm Petri dish containing YT with 1.5% (w/v) agar. Plates were incubated at 37°C overnight.

The white plaques, made by phage which contained inserted cDNAs, were removed as an agar plug, placed into a tube (4ml) of YT and 100μl log phase E. coli TG-1, and allowed to grow overnight at 37°C. The cultures were centrifuged at 1000xg for 10 minutes. The bacterial pellets were discarded, and the phage particles in the supernatant fraction, which contained single stranded DNA, were precipitated by addition of 1/5 volume of 20% (w/v) polyethylene glycol and 2M NaCl. After an incubation of 1 hour on ice, the phage were collected by centrifugation at 10,000xg for 30 minutes at 4°C. The phage were resuspended in a solution of TE containing 1% (w/v) SDS and the samples were extracted with phenol/chloroform. The single stranded DNA was then ethanol precipitated.

DNA sequencing. DNA was sequenced according to the method of Sanger et al. (1977) as outlined in the New England BioLabs technical bulletin. The 17-mer universal primer (2ng) was allowed to anneal with purified, single-stranded M13 DNA (1μg) at 50°C in a sequencing buffer containing 0.01M Tris-HCl, pH 7.5, 5mM $MgCl_2$ and 7.5mM dithiothreitol. Meanwhile, the "enzyme mix" was prepared, containing 6U large fragment of E. coli DNA polymerase and 80μCi (800 Ci/mmol) [α-^{32}P]-dATP in the sequencing buffer described above. The "enzyme mix" was then added to each of the

tubes containing M13 DNA and the annealed primer. For each clone, this mixture of phage, primer and enzyme mix was evenly divided into four tubes labeled "G", "A", "T", and "C". To the "G" tubes for each clone, the "G" sequencing mix was added; to the "A", tubes, "A" sequencing mix and so on. The compositions of the sequencing mixes are as follows: "G", 150μm dideoxy GTP (ddGTP), 1.7μm dATP, 40μM dCTP, 4.2μM dGTP, and 40μM dTTP; "A", 60μm ddATP, 1.7μm dATP, 30μM dCTP, 30μM dGTP and 30μM dTTP; "T", 150μM ddTTP, 1.7μM dATP, 40μM dCTP, 40μM dGTP, and 1.7μM dTTP; "C", 150μM ddCTP, 1.7μM dATP, 4.2μM dCTP, 40μM dGTP and 40μM dTTP; each sequencing mix was in the sequencing buffer described above. The primer-template mixture plus enzyme mix and sequencing mixes were allowed to remain at room temperature for 15 minutes. A chase solution containing 0.25mM deoxynucleotides was added to the sequencing reactions, and the samples remained at room temperature for 15 minutes. The reactions were terminated by the addition of a solution of deionized formamide containing 0.3% (w/v) xylene cyanol FF, 0.3% (w/v) bromphenol blue, and 0.37% (w/v) EDTA, pH 7.0.

The sequencing reactions were subjected to electrophoresis on thin gels (0.04 cm), 40 x 65cm, consisting of 6% (w/v) polyacrylamide, 7M urea and 0.05M TBE. The gels were dried immediately after electrophoresis, and exposed to Kodak XAR film at room temperature. The sequence was analyzed with the aid of the Beckman MicroGenie program.

Heparin-Agarose affinity chromatography. Heparin-Agarose (800μg heparin/ml gel; 4mg) was equilibrated in a buffer of 0.01M Tris HCl, pH 7.5 with 0.010M NaCl. A bed volume of approximately 5ml was used. Approximately 6mg of uteroferrin in the same buffer was applied to the

column. The column was washed with 3 column volumes of the same buffer, and the uteroferrin eluted step-wise with 0.1, 0.25 and 0.5M NaCl in 0.01M Tris HCl, pH 7.5.

Tissue culture techniques. The cell lines employed were as follows: The human erythroleukemia cell line K562 (Lozzio and Lozzio, 1975), the human T-cell line, JURKAT, also known as JM (see Nagasawa et al., 1986), and the Epstein-Barr virus (EBV)-transformed B-cell line 1799ZR1.3 (Smith et al., 1987). The cells were maintained in RPMI 1640 medium with Hepes buffer, supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2mM glutamine and antibiotic solution (10μg/ml penicillin, 10μg/ml streptomycin, 0.25μg/ml amphotericin B). The K562 cells and JURKAT cells were maintained at 37°C in a 5% CO_2 atmosphere; the 1799ZR1.3 cells, at 37°C in a 7% CO_2 atmosphere. Cells were maintained at a density of 2.5 x 10^5-8.0 x 10^5 cells/ml. Only cells that were at least 98% viable as determined by Trypan blue exclusion were used for experiments, except where stated in the text. The normal human leukocytes and leukemic hairy cells (the generous gift of M.A. Gross and R. Weiner) were prepared for cryopreservation after collection (Weiner et al., 1979) and thawed immediately before use.

A solution of 12-tetradecanoylphorbol 13-acetate (TPA) was prepared as a 1.6mM stock in dimethyl sulfoxide and stored frozen. For each experiment, a fresh stock of 10^{-6}M was prepared in the growth medium described above.

Hemin (4 x 10^{-3}M stock) was prepared according to the method of Rutherford and Weatherall (1979). Thirteen milligrams of hemin were dissolved in 200μl of 0.5M NaOH, then neutralized with 250μl 1M

Tris-HCl, pH 7.8 and brought up to 5ml with deionized distilled water. The stock was made fresh and used immediately.

Isolation of RNA from tissue culture cells. RNA was isolated according to the method of Chomczynski and Sacchi (1987). Tissue culture cells were centrifuged at 1500xg for 10 minutes at room temperature. For each 10^6 cells in the cell pellet, 100μl of GTC solution was added (4M guanidinium thiocyanate; 0.025M sodium citrate, pH 7.0; 0.5% (w/v) sarcosyl; 0.1M 2-mercaptoethanol). The sample was vortexed immediately, and the cells broken in a dounce homogenizer. An equal volume of water-saturated phenol was added, followed by an addition of 0.1 volume 2M sodium acetate pH 4.0, and 0.2 volumes of chloroform: isoamyl alcohol, 49:1. The samples were shaken vigorously and placed on ice for 15 minutes. The samples were centrifuged at 10,000xg for 30 minutes. RNA remained in the aqueous phase, DNA was at the interface between the two phases, and most other cellular components were in the organic phase. The aqueous phase was carefully collected, and the RNA precipitated with isopropanol. The RNA pellet was resuspended in GTC, and precipitated with isopropanol a second time. The RNA was then precipitated with ethanol, and the pellet resuspended in RNase-free water.

Electrophoresis of RNA. RNA samples were subjected to electrophoresis in a gel consisting of 1% (w/v) agarose, 0.04M morpholiropropanesulfonic acid (MOPS), pH7.0, 0.01M sodium acetate, 2.2M formaldehyde and 1mM EDTA (Lehrach et al., 1977). RNA samples were dissolved in the above buffer containing 50% (v/v) formamide, 0.5% (w/v)

bromphenol blue and 0.5% (w/v) xylene cyanol FF. RNA was detected by staining with 0.1μg/ml of ethidium bromide for 10 minutes.

Northern blotting. Northern blotting of formaldehyde-agarose gels was carried out according to Maniatis et al. (1982). The gel was soaked in 50mM NaOH, 0.01M NaCl, followed by neutralization in 1.0M Tris-HCl, pH 7.5. Transfer of the RNA to GeneScreen was allowed to proceed by capillary action overnight in 20X SSC. After a brief wash in 0.05M TBE, the blot was UV-irradiated in order to cross-link the RNA to the membrane (Church and Gilbert, 1984).

Preparation of a single-stranded probe for Northern analysis. A [^{32}P]-labeled single-stranded probe which hybridizes to mRNA coding for the human tartrate-resistant acid phosphatase was prepared in the following manner (Church and Gilbert, 1984): Approximately 2.5μg of single stranded M13 DNA containing the coding strand of the clone coding for the human phosphatase were allowed to anneal with 18ng of M13 sequencing primer at 50°C in a buffer containing 0.05M Tris-HCl, pH 8.0 and 0.3M NaCl. After 40 minutes, the deoxynucleotides dCTP, dGTP and dTTP were added to a concentration of 0.066mM, in a solution containing 5mM $MgCl_2$, 0.01M 2-mercaptoethanol, 0.3mg/ml BSA and 100μCi (800 Ci/mmol) [α-^{32}P]-dATP. After 40 minutes at room temperature, the reaction was terminated by the addition of a solution of 50% (v/v) formamide, 0.03% (w/v) xylene cyanol FF, 0.3% (w/v) bromophenol blue and 0.037% (w/v) EDTA. The sample was subjected to electrophoresis in a 6% (w/v) polyacrylamide gel with 7M urea in 0.05M TBE in order to separate the radiolabeled probe for the M13 template. The gel was placed in contact with Polaroid Type 57 film in order to determine how far the

radiolabeled probe had migrated. The radioactive band was cut from the gel, crushed with a glass rod, and then mixed with hybridization solution of 1M sodium phosphate buffer, pH 7.2, 7% (w/v) SDS, 1mM EDTA and 1% (w/v) BSA. The probe was shaken at 50°C for 2 hours. Hybridization was then carried out for 16 hours at 65°C. The blots were washed with 1 liter (5 x 200ml) of a wash buffer consisting of 0.076M sodium phosphate buffer pH 7.2 with 1mM EDTA and 0.1% (w/v) SDS at 65°C. Autoradiography was performed at -70°C with Kodak XAR film and DuPont Cronex Lightning Plus intensifying screens.

The β-actin probe for Northern blotting. A human β-actin cDNA (Gunning et al., 1983; Ponte et al., 1984) in the Okayama-Berg expression vector (Okayama and Berg; 1983) was used as an internal control in Northern analysis. The cDNA insert was isolated as a BamH I restriction fragment. The β-actin cDNA was radiolabeled by the random primer extension method described previously. Hybridization and washing conditions were as described for the tartrate-resistant acid phosphatase probe.

Preparation of cell lysates. Cells in culture were centrifuged at 1500xg for 10 minutes at room temperature. The culture medium was frozen at -70°C. The cells were lysed in the lysis buffer (described in Chapter 2) which had been used for isolation of the acid phosphatase from human spleen. The cells were broken in a dounce homogenizer, then centrifuged at 14,000xg for 10 minutes. The pellet was discarded, and the supernatant fraction frozen immediately at -70°C.

Measurement of acid phosphatase activity. Acid phosphatase activity in both the cell lysates and the culture medium was determined in the

presence of 0.1M tartrate and 0.1M 2-mercaptoethanol at pH 5.3 with the substrate p-nitrophenylphosphate as described in Chapter 2.

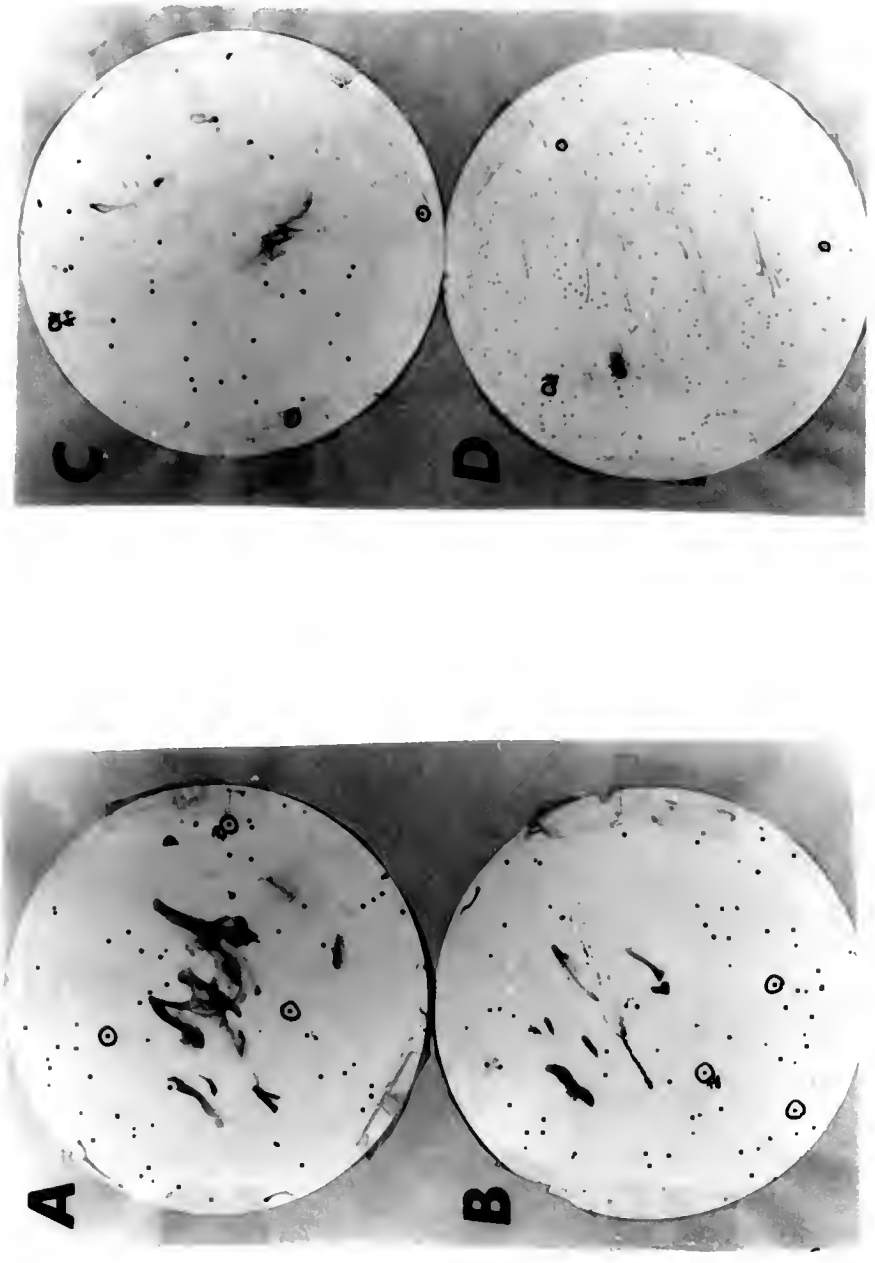
Results

The Identity of the Positive Clones Obtained by Immunoscreening the Human Placenta Library

When polyclonal antisera raised against porcine uteroferrin were used to immunoscreen 3 x 10^5 plaques of the human placenta cDNA library in lambda gtll, 12 positive clones were obtained. Each of these clones produced a fusion protein recognized by three independent lots of rabbit antisera. Filters from the immunoscreening are shown in Fig. 3-3. Of the twelve positive clones, three could be described as generating very strong immunoreactive fusion proteins, seven generated moderate reactions, one gave a weak reaction, and one appeared to be a false positive but was isolated nevertheless. Each of these lambda gtll clones except the suspected false positive (clone HP 1.1) continued to give rise to immunoreactive fusion proteins throughout the course of plaque purification.

The three very strong positive clones, HP 5.1, HP 5.2 and HP 6.1 were chosen for further analysis. The lambda gtll DNA was purified from these clones and treated with EcoR I, and the sizes of the cDNA inserts were determined (Fig. 3-4A). Clone HP 6.1 was approximately 1400 base pairs (bp); clone HP 5.2, 1200 bp; and HP 5.1, 1000 bp. When the insert HP 6.1 was radiolabeled by the random primer extension method, it hybridized to both HP 5.1 and HP 5.2 (Fig. 3-4B), as determined by Southern blot analysis carried out at very high stringency. This same probe hybridized to DNA from plaques produced by 10 of the 11 remaining positive clones (data not shown). Only clone HP 1.1, the suspected

Fig. 3-3 Nitrocellulose filters containing immunoreactive fusion proteins produced by recombinant lambda gtll phage from a human placenta cDNA library which was screened with anti-uteroferrin antibodies. Each filter contains fusion proteins from approximately 2000 recombinant phage, which had been enriched for positive clones by one round of plaque purification. The filters were incubated with rabbit antibodies against porcine uteroferrin, followed by goat-anti-rabbit conjugated to horseradish peroxidase. Immunoreactive fusion proteins were visualized by incubation with substrate [0.015% (w/v) H_2O_2] and coupling dye (4-chloro-1-naphthol, 0.33mg/ml). A, clone HP5.2; B, clone HP5.2, screened with antisera from a different rabbit; C, clone HP6.1; D, clone HP5.1. The filters in panels C and D were tested with the same antisera used for the filter in panel A.


C
D
A
B

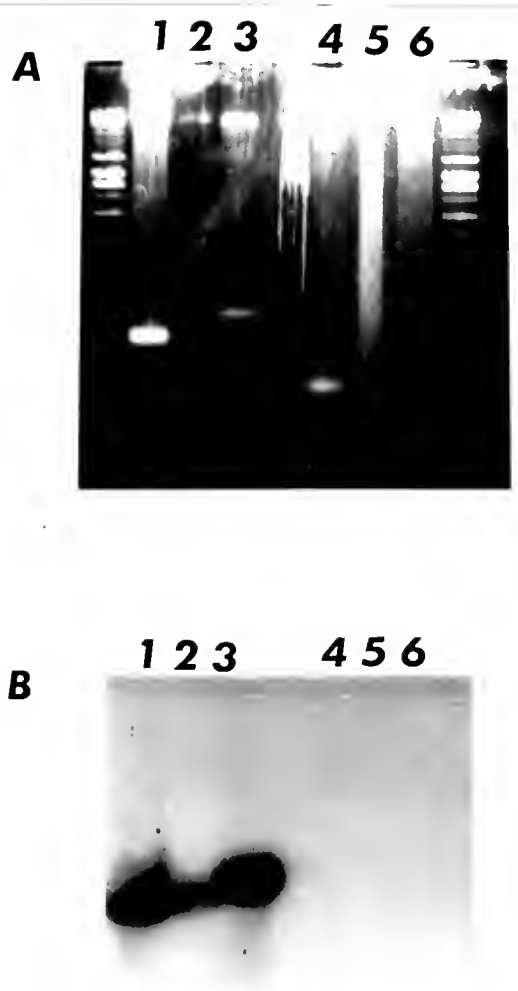


Fig. 3-4 Agarose gel electrophoresis of DNA from six positive clones obtained by immunoscreening human placenta and mouse spleen cDNA libraries and Southern analysis of the clones probed with HP 6.1. A, Approximately 20μg of lambda gtll DNA containing the following cDNA inserts were digested with EcoR I and subjected to electrophoresis in a 1% (w/v) agarose gel: HP 5.1 (lane 1), HP 5.2 (lane 2), HP 6.1(lane 3), MS 4.4 (lane 4), MS 5.1 (lane 5) and MS 6.1 (lane 6). DNA was visualized with ethidium bromide. The cDNA inserts are the lower, less intense bands of varying size. B, Approximately 1μg of lambda gtll DNA containing the same cDNA inserts were treated as in panel A. Lanes 1-6 contain the same samples as described above. The DNA was blotted onto Gene-Screen and probed with radiolabeled HP 6.1 insert as described in "Materials and Methods". Autoradiography was carried out for 10 minutes (lanes 1-3) or 16 hours (lanes 4-6).

false positive, failed to hybridize to the radiolabeled cDNA insert HP 6.1.

Southern blots of the positive clone HP 6.1 in pUC19 and Msp I restriction fragments of that cDNA, both in pUC19 and as isolated insert, were probed with radiolabeled redundant oligonucleotides which code for porcine uteroferrin (see Fig. 3-2 for the sequences of the oligonucleotides). Probe KM29 did not hybridize to the cDNA HP 6.1. However, under conditions of fairly high stringency, oligonucleotide KM28, which codes for amino acids 11-16 of uteroferrin, hybridized specifically to a 400 bp Msp I fragment of clone HP 6.1 (see Fig. 3-5).

Taken together, these data indicated that clone HP 6.1 coded for the human tartrate-resistant acid phosphatase. A cDNA of 1300 bp could entirely encode a protein of apparent molecular weight 34,000 (about 300 amino acids). Three different lots of monospecific antisera raised against porcine uteroferrin consistently and specifically recognized fusion proteins from 11 different cDNA clones which were known to hybridize to HP 6.1. Finally, an oligonucleotide coding for porcine uteroferrin hybridized specifically to HP 6.1.

The cDNA insert of clone HP 6.1 was digested with Alu I and Hae III. The results of this digest are shown in Fig. 3-6. Each digest was cloned into the sequencing vector M13mp19 ("shotgun" cloning), and clones were chosen at random and sequenced. When the sequence of clone HP 6.1 was analyzed by the Beckman MicroGenie program, it was revealed that this clone actually codes for human fibronectin rather than a uteroferrin-like polypeptide. Clone HP 6.1 covers nucleotides 2324-3798 (1475 bp) of human fibronectin (Kornblihtt et at., 1984) which codes for

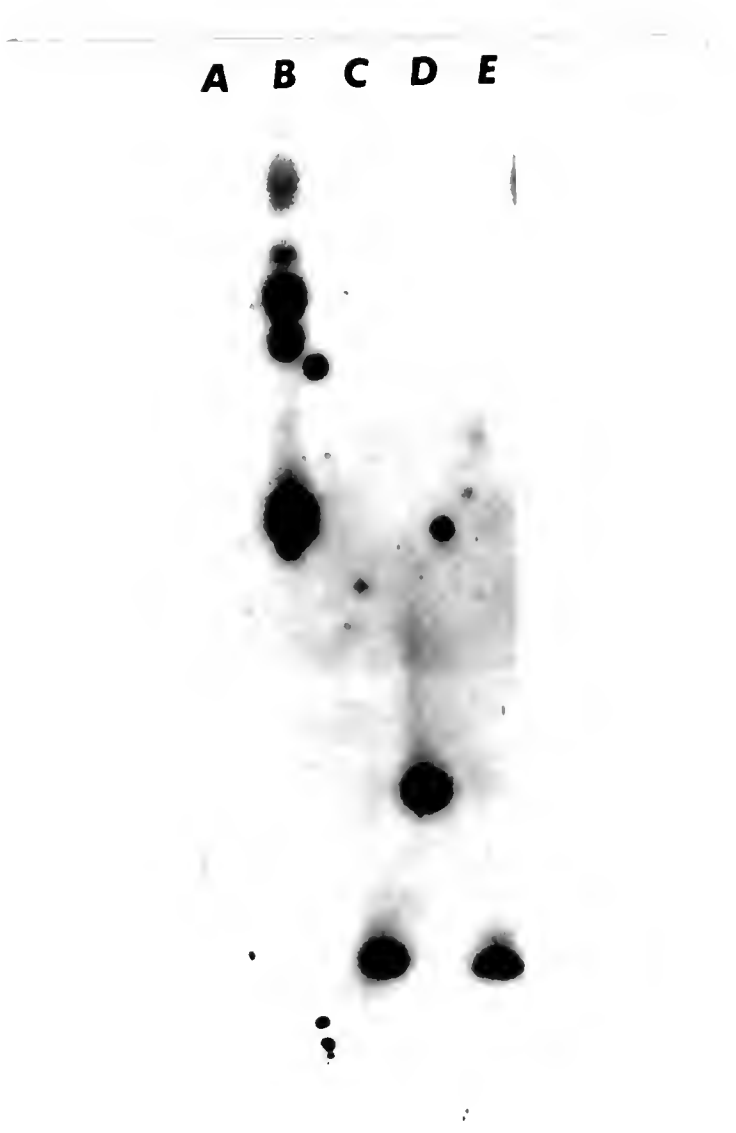


Fig. 3-5 Southern blot analysis of clone HP 6.1 with radiolabeled oligonucleotide KM28. Plasmid pUC19 containing cDNA insert HP 6.1 (1μg) was cut with EcoR I and/or Msp I and subjected to electrophoresis on a 1% (w/v) agarose gel and transferred to Gene-Screen as described in "Materials and Methods". The blot was probed with radiolabeled KM28, which codes for amino acids 11-16 of porcine uteroferrin. The hybridization and washing conditions are explained in the text. A, pUC19 (1μg). B, pUC19 containing the cDNA insert HP 6.1. C, Msp I digest of pUC19 containing the cDNA insert HP 6.1. D, EcoR I digest of pUC19 containing the cDNA insert HP 6.1. E, Msp I digest of the insert HP 6.1, which had been isolated from an EcoR I digest of pUC19 containing the cDNA and electroluted from a 1% (w/v) agarose gel. The plasmid pUC19 contains 13 Msp I sites, the cDNA insert contains 2 Msp I sites.

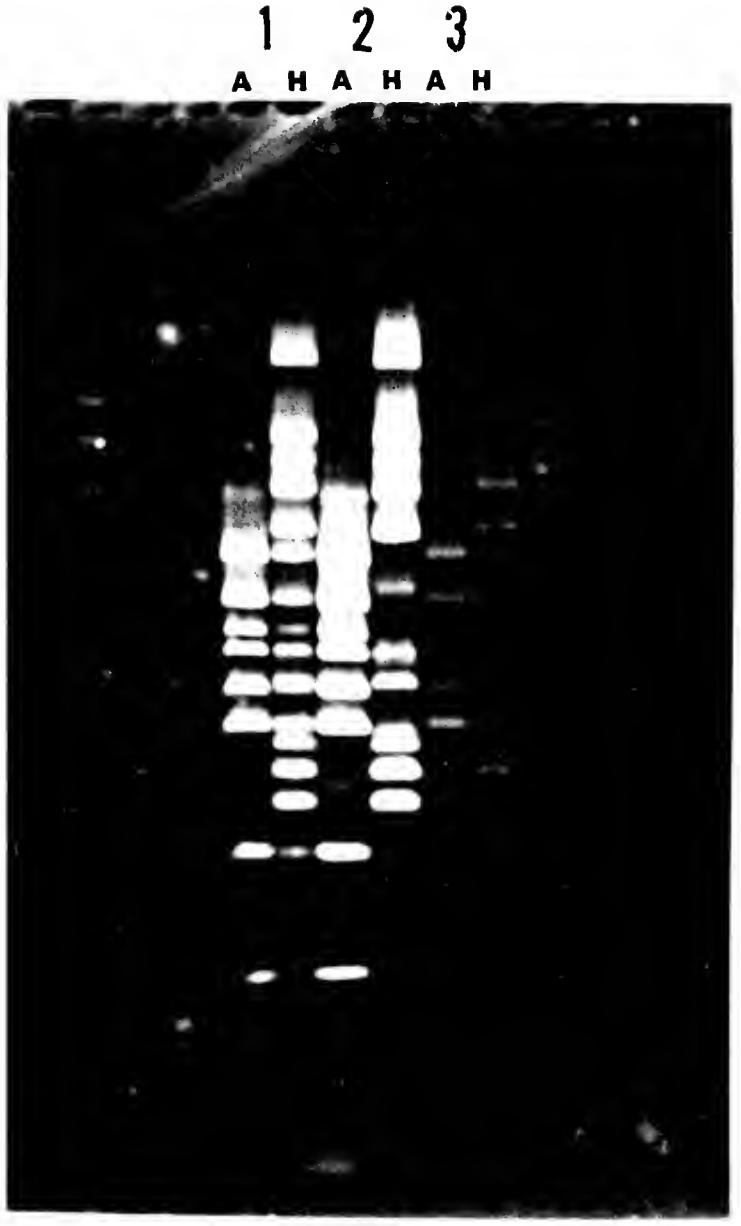


Fig. 3-6 Polyacrylamide gel electrophoresis of clone HP 6.1 restriction fragments obtained by digestion with Alu I and Hae III. Approximately 2μg of HP 6.1 cDNA insert were digested with Alu I (A) or Hae III (H) as described in "Materials and Methods". The insert was obtained from pUC19 clone HP 6.1-3 (lanes 1A and H), pUC19 clone HP 6.1-9 (lanes 2A and H) and lambda gt11 clone HP 6.1 (lanes 3A and H). The digests were subjected to electrophoresis in a 6% (w/v) acrylamide gel and the DNA visualized with ethidium bromide.

amino acids 1709-2201 of the mature protein (see Fig. 3-7; Kornblihtt et al., 1985). Furthermore, nucleotides 3660-3676 of human fibronectin match probe KM28 (which codes for amino acids 11-16 of uteroferrin) at 14 out of 17 residues (Fig. 3-2).

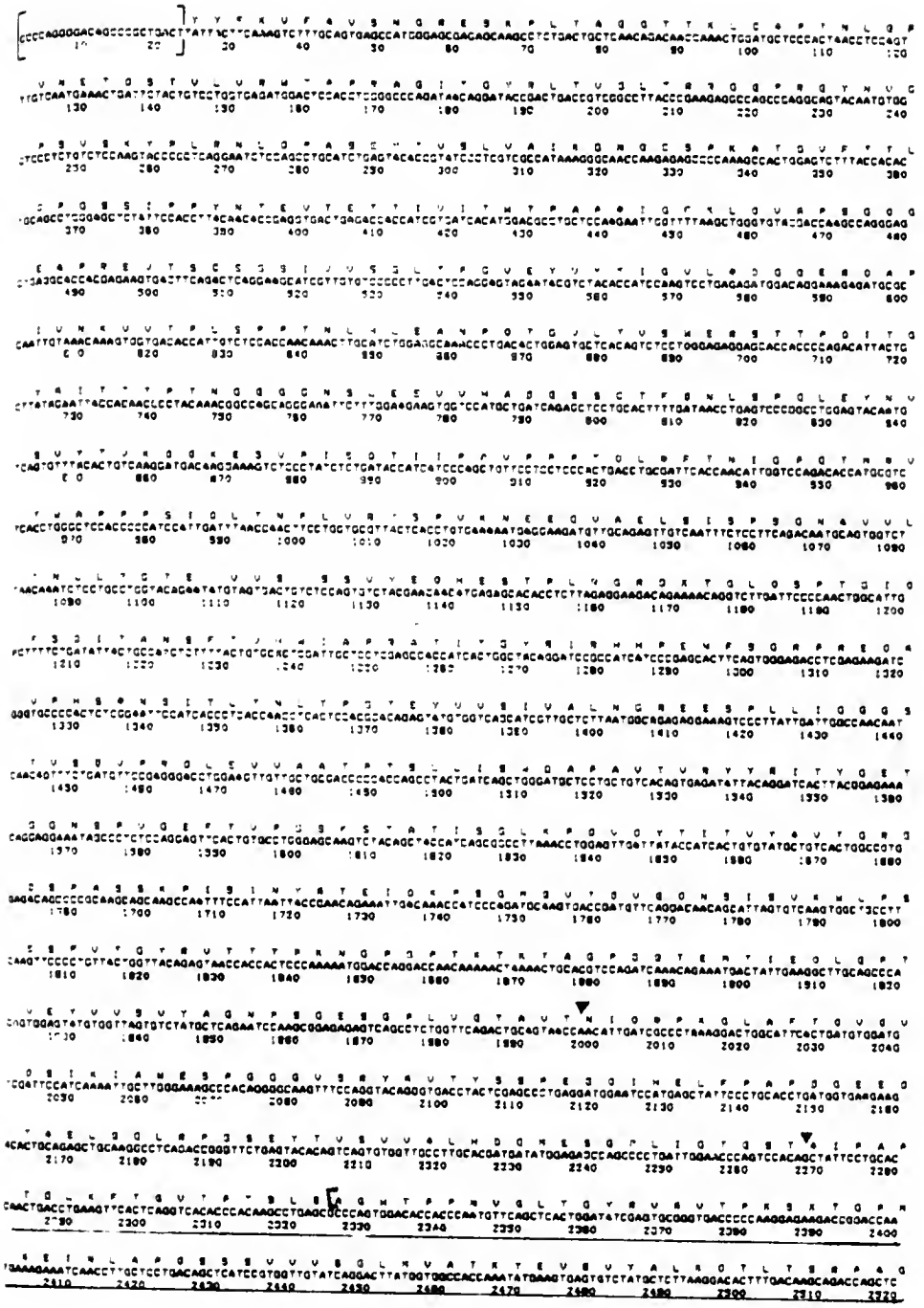
Dot Blot Analysis of Uteroferrin and Fibronectin with Anti-Uteroferrin Antibodies

Uteroferrin, human fibronectin, leucine aminopeptidase and copper/zinc superoxide dismutase were bound to nitrocellulose and tested for cross-reactivity with rabbit polyclonal antibodies raised against porcine uteroferrin. Figure 3-8 reveals that the polyclonal antibodies cross-reacted with human fibronectin as well as with porcine uteroferrin, with binding apparent down to 0.1ng of protein. There was no detectable binding to 1μg samples of leucine aminopeptidase or superoxide dismutase under identical conditions. The four monoclonal antibodies discussed in detail in Chapter 2 were then tested for binding to human fibronectin. Monoclonal antibodies 6.21.2 and 5.127.3 cross-reacted weakly human fibronectin (detection limit 100ng), while monoclonal antibodies 6.22.1 and 13.122 did not.

Results from Screening of the Mouse Spleen cDNA Library

About 2.1 x 10^5 plaques from a mouse spleen cDNA library in lambda gt11 were screened with the anti-uteroferrin polyclonal antibodies. Seventeen positive clones were identified. Two clones generated immunoreactive fusion proteins rated as very strong; seven were rated moderately strong, six were weakly positive, and two were suspected to be false positives. The two strongest positive clones, MS 5.1 and MS 6.1, and one moderately positive clone, MS 4.4 were chosen for further study. The cDNA inserts were excised with EcoR I and subjected to

Fig. 3-7 The nucleotide and inferred amino acid sequence of clone HP 6.1, which codes for human fibronectin. The nucleotide sequence of a full-length cDNA coding for human fibronectin is shown below, and the region which matches clone HP 6.1 is underlined. The inferred amino acid sequence of human fibronectin is listed above, with the deduced amino acid sequence of clone HP 6.1 in brackets. The cDNA sequence and inferred amino acid sequence are from Kornblihtt et al. (1985, 1986).



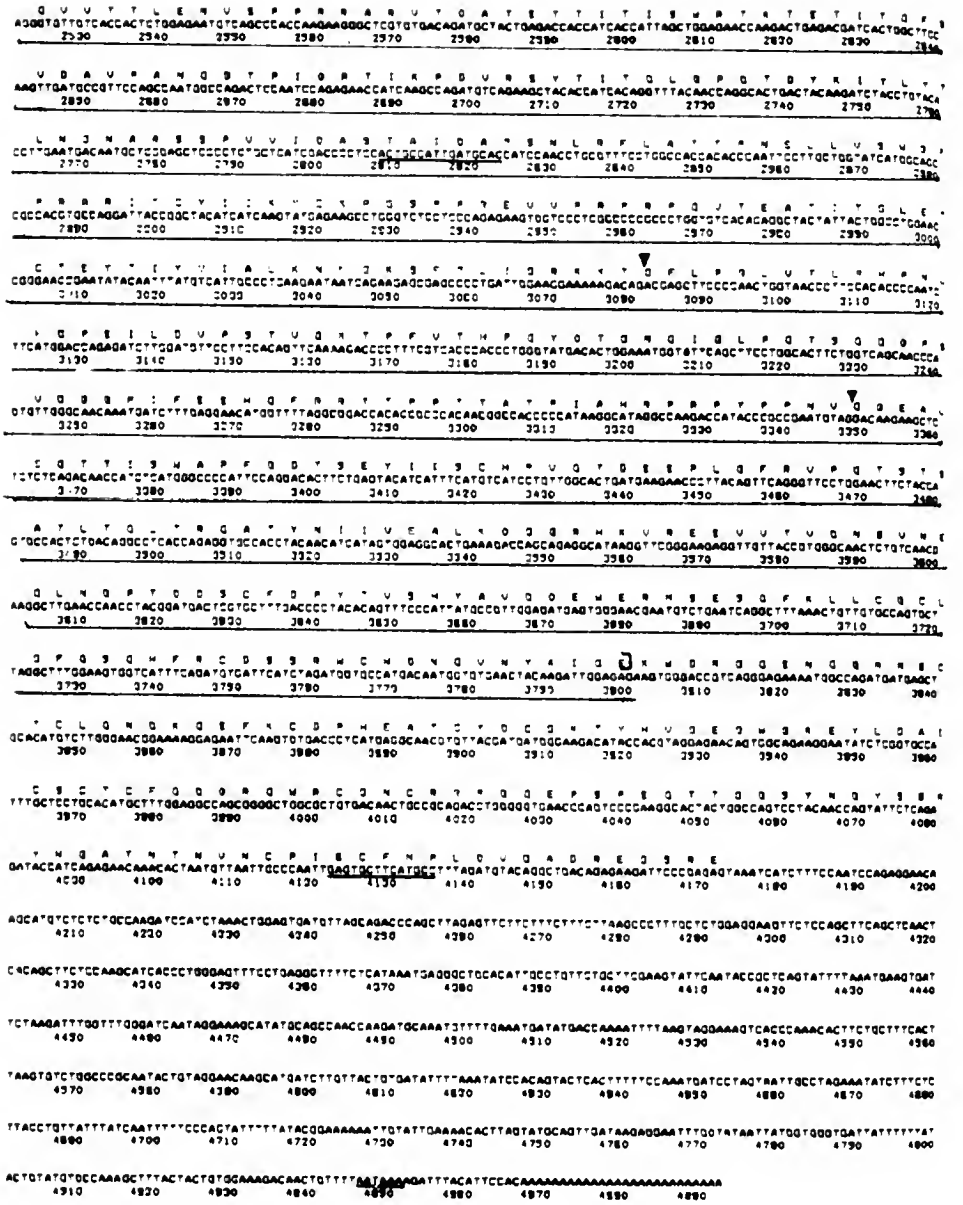
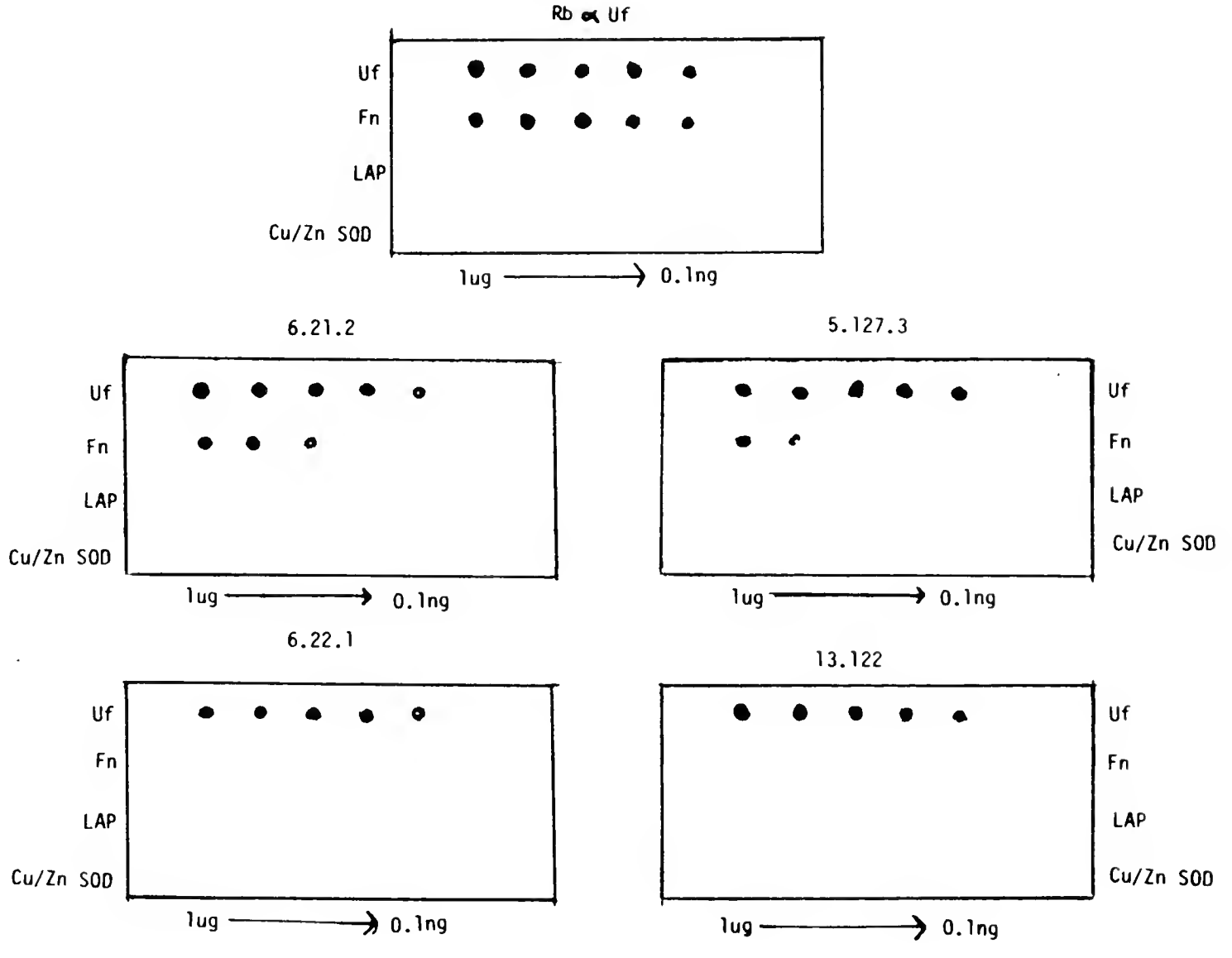


Fig. 3-7--continued

Fig. 3-8 Dot blot analysis of the binding of polyclonal and monoclonal antibodies against porcine uteroferrin to uteroferrin and human fibronectin. Serial ten-fold dilutions of protein, from 1 μg/ 1 to 0.1ng/μl, were bound to nitrocellulose filters (1 1 dots). The proteins employed were Uf, uteroferrin; Fn, fibronectin; LAP, leucine aminopeptidase; and Cu/Zn SOD, copper/zinc superoxide dismutase. After additional protein binding sites on the nitrocellulose were blocked with BSA, the blots were incubated with the source of antibody indicated: Rb $\propto$ Uf, rabbit anti-uteroferrin 1/100 dilution; 6.22.1, that monoclonal antibody, 10μg/ml; 6.21.2, 10μg/ml; 5.127.3, 8μg/ml; 13.122, 8μg/ml. The appropriate secondary antibody (goat anti-rabbit or goat anti-mouse) was incubated with the blots at the manufacturer's recommended dilution. Immunoreactive proteins were detected with H_2O_2 (0.015%, v/v) and 4-chloro-1-napthol (0.33mg/ml). This figure is a reproduction of the original dot blot, which had faded with time.


Rb ∝ Uf
Uf
Fn
LAP
Cu/Zn SOD
1ug
0.1ng
6.21.2
5.127.3
6.22.1
13.122

electrophoresis on a 1% (w/v) agarose gel (Fig. 3-4A). The approximate sizes of the cDNA clones were as follows: MS 4.4, 575 bp; MS 5.1, 240 bp; and MS 6.1, 300 bp. These cDNA inserts were also on the Southern blot in Fig. 3-4B. Note that HP 6.1, which codes for human fibronectin, did not hybridize to these cDNA inserts.

The largest cDNA clone, MS 4.4, was chosen for further study. An agarose gel of an EcoR I digest of a larger quantity of DNA from this clone revealed the presence of an additional insert of about 250 bp (data not shown). Both the 575 bp and 250 bp cDNAs were subcloned into M13mp18 and sequenced. The sequences of the cDNAs showed no homology to fibronectin, nor were they homologous to any other DNA sequences in GenBank. The inferred amino acid sequences of the clones showed no similarity to the amino acid sequence of porcine uteroferrin. No further studies were carried out with the mouse cDNA clones obtained by immunoscreening.

Results from Screening the Porcine Uterine Endometrium cDNA Library

Approximately 3 x 10^5 plaques from a porcine uterine endometrium library (the generous gift of George Baumbach) were screened with the anti-uteroferrin antibodies. No positive clones were obtained. However, when this library was immunoscreened with antibodies raised against the uteroferrin-associated protein mentioned in Chapters 1 and 2, several positive clones were obtained (M.K. Murray, unpublished results). It is not clear why a cDNA insert coding for porcine uteroferrin could not be isolated from this library by immunoscreening.

The Molecular Cloning of a cDNA Coding for the Human Type 5, Tartrate-resistant Acid Phosphatase

Approximately 3 x 10^5 plaques from the human placenta cDNA library were screened with two short cDNA probes which code for porcine uteroferrin (Fig. 3-1). These cDNA clones, 13.1 and 4a3, were obtained by Rosalia Simmen (Ohio State University) upon immunoscreening a porcine uterine endometrium library. The synthesis of the library and isolation of the positive clones are described elsewhere (Simmen et al., 1988). Upon screening the human placenta cDNA library with uteroferrin cDNA 4a3, eight positive clones were identified. Two of these positive clones 2a, and 6a, were rated as strong positives; three as moderately strong positives; and three as weak positives. When these positive clones were rescreened with cDNA 4a3, only the two strong positives hybridized to the probe. The cDNA 13.1, when used as a probe, hybridized to both of these strong positives, 2a and 6a (Fig. 3-9). The cDNA inserts were excised from lambda gt11 clones 2a and 6a with EcoR I and subjected to electrophoresis on a 1% (w/v) agarose gel. The cDNA 2a appeared to be approximately 900 bp; the cDNA 6a, about 1400 bp (Fig. 3-10).

The cDNA clones 2a and 6a were digested with Alu I, Hae III, Msp I and Rsa I, and the digests subjected to electrophoresis on 6% (w/v) polyacrylamide gels (Fig. 3-11). The fragments larger than 100 bp were electroeluted from the gels, and subcloned individually into M13mp18. The subcloning and sequencing strategy is shown in Fig. 3-12. The restriction fragments that were sequenced and their locations in clone 6a are listed in Table 3-1.

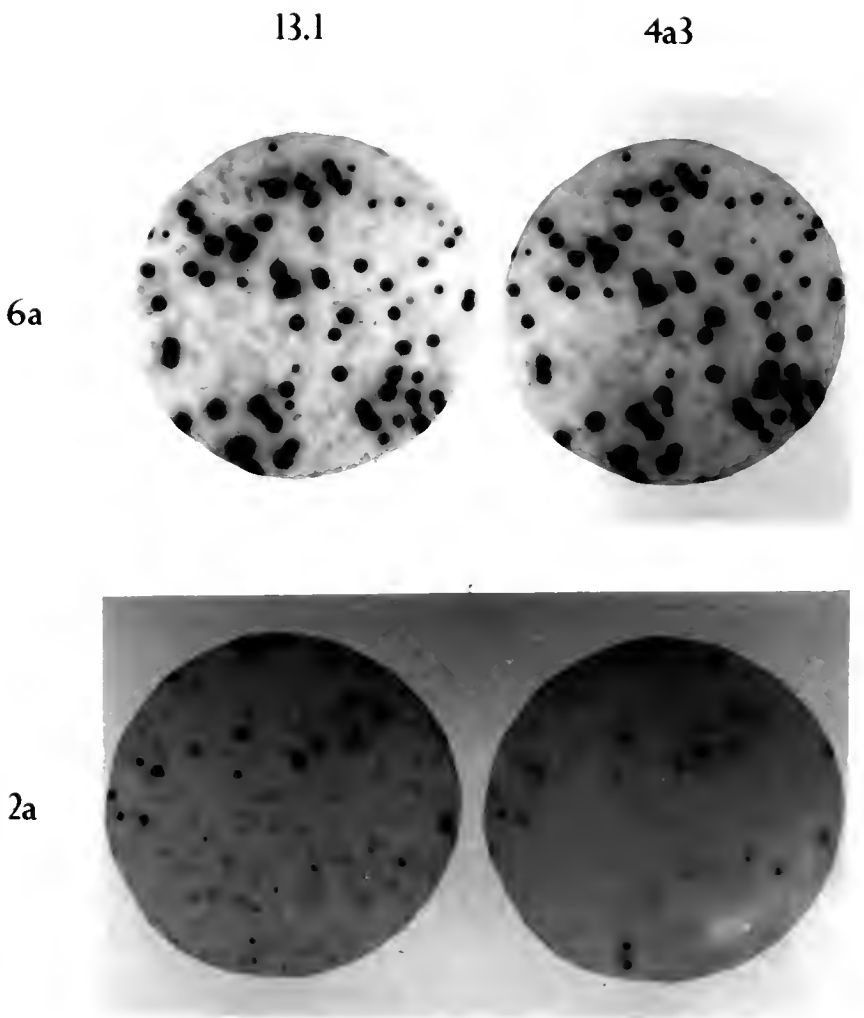


Fig. 3-9 Two positive clones, 2a and 6a, identified by screening the human placenta cDNA library with the cDNAs coding for porcine uteroferrin. Duplicate filters were obtained containing DNA from approximately 1 x 10^3 plaques (6a, top) or 600 plaques (2a, bottom) at an intermediate stage in plaque purification of the two positive clones (see "Materials and Methods"). The filters were hybridized to radiolabeled cDNAs coding for 13.1 (left) or 4a3 (right) as described in the text.

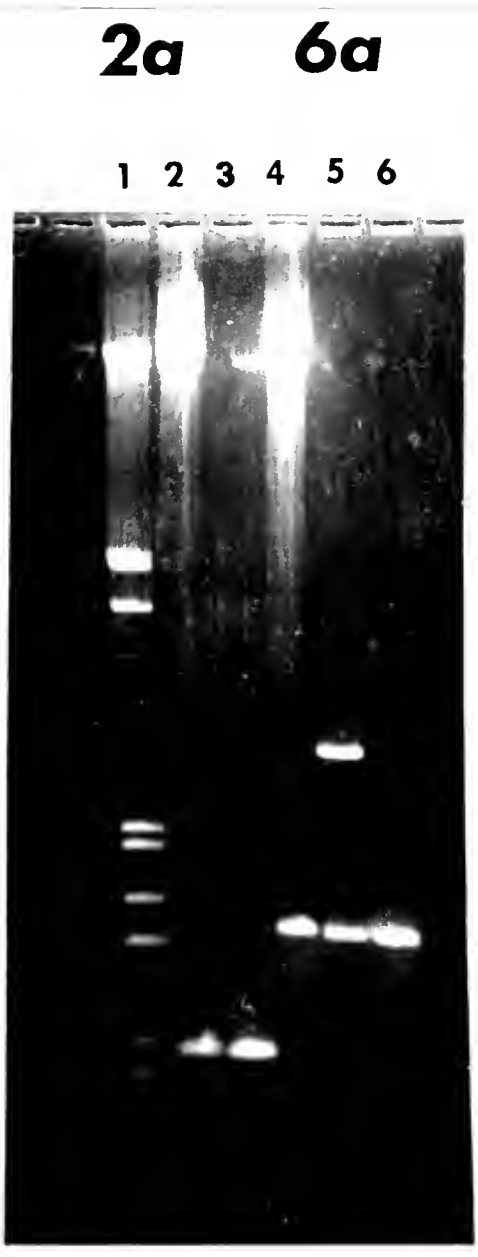
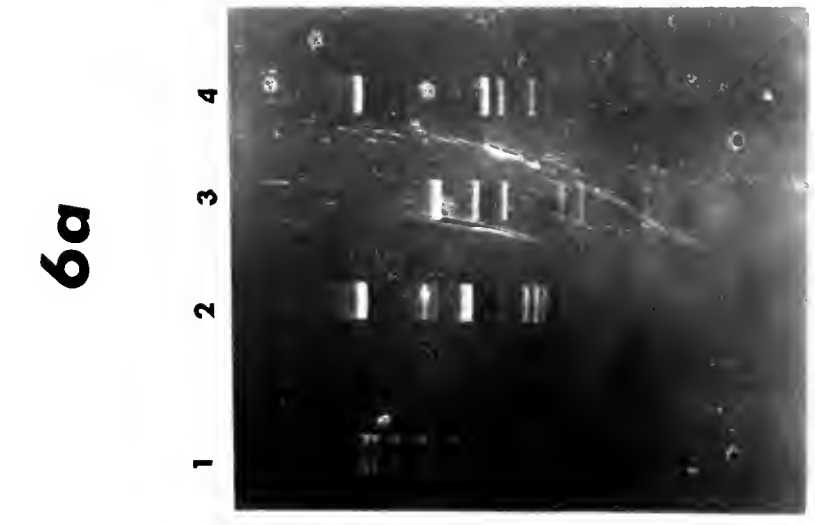
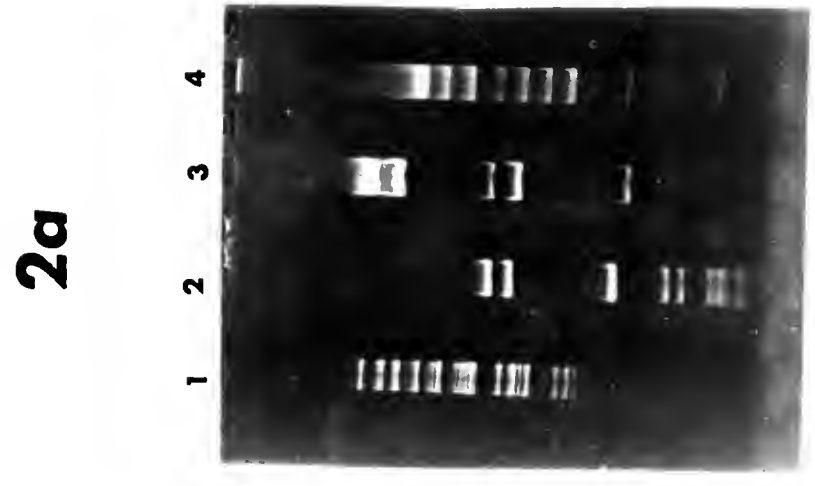


Fig.3-10 Agarose gel electrophoresis of EcoR I digests of DNA from two positive clones, 2a and 6a. DNA isolated from lambda gtll positive clones (10μg; lanes 2 and 4) and a pUC19 subclone (2μg; lane 5) which had been digested with EcoR I were subjected to electrophoresis on a 1% (w/v) agarose gel. Isolated cDNA inserts which had been electroeluted from a similar gel (100ng) were also subjected to electrophoresis on this agarose gel (lanes 3 and 6). Molecular weight markers (lane 1) were EcoR I and Hind III digests of phage DNA (top to bottom; 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1340, 983, 831 and 564 bp). The sizes of cDNA inserts 2a (lanes 2 and 3) and 6a (lanes 4-6) were estimated to be 900 and 1400 bp, respectively.

Fig.3-11 Polyacrylamide gel electrophoresis of the restriction fragments obtained by the digestion of clones 2a and 6a with Alu I, Hae III and Msp I. Clone 2a (left) was digested with Alu I (incomplete digest, lane 1) Hae III (lane 2) or Msp I (lane 3). Molecular weight markers (lane 4) consisted of an Msp I digest of pUC19 (from top to bottom; 501, 489, 331, 242, 190, 147, 111-110 doublet, 67 and 34 bp, respectively). Clone 6a (right) was digested with Alu I (lane 2), Hae III (lane 3) or Msp I (lane 4). Molecular weight markers (lane 1) were the same as described for 2a. The samples were subjected to electrophoresis in a non-denaturing 6% (w/v) polyacrylamide gel. The restriction fragments were visualized with ethidium bromide.


6a
4
3
2
1


2a
4
3
2
1

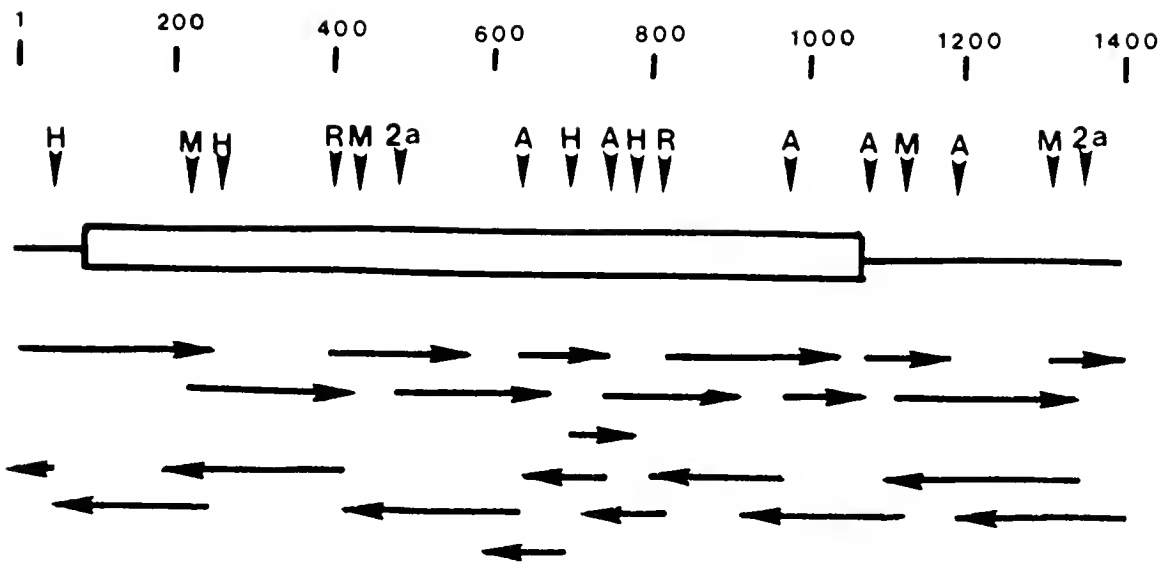


Fig.3-12 Subcloning and sequencing strategy for clone 6a, the cDNA encoding the human Type 5, tartrate-resistant acid phosphatase. The restriction map displays only relevant restriction endonuclease sites. The solid bar represents the protein coding region, the thin line the untranslated sequences. The direction and extent of the sequence determinations are shown by horizontal arrows. A, Alu I; H, Hae III; M, Msp I, R, Rsa I; 2a, the limits of the shorter cDNA clone 2a.

TABLE 3-1

The clones employed for generation of the complete sequence of the human tartrate-resistant acid phosphatase

Clone	Strand	Nucleotides
2a1,2a3,2a4	L	1215-1367
2a2	U	482-657
6a1,6a5	U	1-89
6a2, 6a3, 6a4	L	1298-1412
a1.1,a1.3,a1.4,r2.3,r2.4,r2.6	U	1-220
a1.2, a3.3	L	1083-1173
a1.4, h4.2, sh6, r2e, r2k	U	449-621
a2.1	U	778-916
a2.3	L	802-978
a2.6	L	913-972
a3.1	L	987-1076
a3.4	U	1002-1087
a3.5	L	633-758
a3.7	L	986-1081
h1.1	U	1118-1281
h1.2	L	1269-1412
h1.4(6a)	U	239-406
h2.1, h3.2, h3.4	U	808-949
h2.2, h3.3(2a)	L	895-1011
h2.1, h2.2, h2.3, h3.3(6a)	L	105-220
h2.6	L	77-196
h3.1, h3.3	L	897-993
h3.1a	L	554-612
h3.1b	U	697-764
h3.5	L	896-1012
h3.6	L	118-226
h4.2	L	1-76
h5.1	L	1060-1091
m1.2, m1.3, m1.5	L	964-1117
m1.3(6a)	U	554-604
m2.1, m2.11, sm1, sm5	L	230-418
m2.2	L	112-230
m1.2, m2.1, m2.2, m2.3	U	482-639
m2.4	L	992-1112
m3.1	L	601-721
m4.2, m4.3	U	1309-1412
r2a	U	407-581
r2c, r2d, r2h	L	204-406
r2ha1	L	731-764
r2ha2	L	771-805
r2hd	U	396-477
ralu a,c,i	U	763-823
ralu b, d, g, h	U	640-660
ralu e	L	666-752
ralu f	U	672-756
sa5	U	755-919

Clones which generated coding-strand sequence are designated U (upper strand); those which generated sequence from the anti-sense strand, L (lower strand). Nucleotides refers to the position of the clone in the complete sequence.

cDNA Sequences

Figure 3-13 shows the 1412 nucleotide sequence of the clone 6a, and the inferred amino acid sequence of the human tartrate-resistant acid phosphatase. The shorter clone, 2a, is identical to nucleotides 482-1367 of clone 6a.

The translational initiation site can be assigned to either of two in-frame ATG codons, both of which lie downstream of an in-frame termination codon, TGA, at nucleotides 70-72. The 5′ untranslated sequence is 93 bp, followed by an open reading frame of 969 bp corresponding to a protein of 323 residues. The codon specifying proline 304 is followed immediately by the translational termination codon TGA at nucleotides 1063-1065. The 3′ untranslated sequence is 347 nucleotides in length, with residues 1381-1386 composing a potential polyadenylation signal AATAAA, which lies 21 residues upstream of the presumed poly(A) tail.

Characteristics of the Deduced Amino Acid Sequence

The first amino acid of the mature protein is assigned as the aspartate coded by nucleotides 151-153. this assignment was determined by application of the (-3, -1) rule of Von Heijne (1983, 1986), but remains tentative since the N-terminal sequence of the human enzyme has not been established.

The mature human tartrate-resistant acid phosphatase most probably consists of 304 amino acids, with an unglycosylated relative molecular mass of 34,193. This enzyme contains two potential attachment sites for N-linked oligosaccharides. The sequence asparagine-valine-serine occurs twice, at amino acids 97-99 and 128-130 (Fig. 3-13).

Fig.3-13 The nucleotide sequence of the cDNA clone 6a and the deduced amino acid sequence of the human Type 5, tartrate-resistant acid phosphatase. The initiation and termination codons are underlined. The putative signal peptidase cleavage site is indicated with an arrow. The two potential attachment sites for N-linked oligosaccharides are boxed. The putative polyadenylation signal (AATAAA) is also underlined.

70
CGCCACGCAATAAAGCCTCACGCACCCGCACTTCTACTCTAGAGCCCACCAGCCTCTCAGAGCCTCCGCT

71 150
GACTGCCCTGTCTCTCCCCCTCG ATG GAC ATG TGG ACC GCG CTG CTC ATC CTG CAA GCC TTG TTG CTA CCC TCC CTG GCT
51
M D M W T A L L I L Q A L L L P S L A

151 225
GAT GGT GCC ACC CCT GCC CTG CGC TTT GTA GCC GTG GGT GAC TGG GGA GGG GTC CCC AAT GCC CCA TTC CAC ACG
1 25
D G A T P A L R F V A V G D W G G V P N A P F H T

226 300
GGC CCG GAA ATG GCC AAT GCC AAG GAG ATC GCT CGG ACT GTG CAG ATC CTG GGT GCA GAC TTC ATC CTG TCT CTA
26 50
G P E M A N A K E I A R T V Q I L G A D F I L S L

301 375
GGG GAC AAT TTT TAC TTC ACT GGT GTG CAA GAC ATC AAT GAC AAG AGG TTC CAG GAG ACC TTT GAG GAC GTA TTC
51 75
G D N F Y F T G V Q D I N D K R F Q E T F E D V F

376 450
TCT GAC CGC TCC CTT CGC AAA GTG CCC TGG TAC GTG CTA GCC GGA AAC CAT GAC CAC CTT GGC AAT GTC TCT GCC
76 100
S D R S L R K V P W Y V L A G N H D H L G N V S A

451 525
CAG ATT GCA TAC TCT AAG ATC TCC AAG CGC TGG AAC TTC CCC AGC CCT TTC TAC CGC CTG CAC TTC AAG ATC CCA
101 125
Q I A Y S K I S K R W N F P S P F Y R L H F K I P

526 600
CAG ACC AAT GTG TCT GTG GCC ATT TTT ATG CTG GAC ACA GTG ACA CTA TGT GGC AAC TCA GAT GAC TTC CTC AGC
126 150
Q T N V S V A I F M L D T V T L C G N S D D F L S

601 675
CAG CAG CCT GAG AGG CCC CGA CTA ACT GCC CGC ACA CAG CTG TCC TGG CTC AAG AAA CAG CTG GCG GCG GCC AGG
151 175
Q Q P E R P R L T A R T Q L S W L K K Q L A A A R

676 750
GAG GAC TAC GTG CTG GTG GCT GGC CAC TAC CCC GTG TGG TCC ATA GCC GAG CAC GGG CCT ACC CAC TGC CTG GTC
176 200
E D Y V L V A G H Y P V W S I A E H G P T H C L V

751 825
AAG CAG CTA CGG CCA CTG CTG GCC ACA TAC GGC GTC ACT GCC TAC CTG TGC GGC CAC GAT CAC AAT CTG CAG TAC
201 225
K Q L R P L L A T Y G V T A Y L C G H D H N L Q Y

826 900
CTG CAA GAT GAG AAT GGC GTG GGC TAC GTG CTG AGT GGG GCT GGG AAT TTC ATG GAC CCC TCA AAG CGG CAC CAG
226 250
L Q D E N G V G Y V L S G A G N F M D P S K R H Q

901 975
CGC AAG GTC CCC AAC GGC TAT CTG CGC TTC CAC TAT GGG ACT GAA GAC TCA CTG GGT GGC TTT GCC TAT GTG GAG
251 275
R K V P N G Y L R F H Y G T E D S L G G F A Y V E

976 1050
ATC AGC TCC AAA GAG ATG ACT GTC ACT TAC ATC GAG GCC TCG GGC AAG TCC CTC TTT AAG ACC AGG CTG CCG AGG
276 300
I S S K E M T V T Y I E A S G K S L F K T R L P R

1051 1145
CGA GCC AGG CCC TGAACTCCCATGACTGCCCAGCTCTGAGGCCCCATCTCCACTGTTGGGTGGCTGGCCTGCCGGGACCCTGCTCACAGGCACCC
301 304
R A R P

1146 1245
TTTTCCTCCAACCTCTGCCCCTGCAGCAGGGCAGGAAGGGCAAACACAGCTGATGAACTGTGGTGCCACATGACCTTGTGGCACAGATGCCACTATGTCA

1246 1345
ACACACATGGACATGTGTCCAGCACAGTGTATGCTCTTGGTCTGGCTCACCCTTTGCTGACTTCCCGGGTGCAATGGGGCAGGCAGGCAGGCAAAGCTTC

1346 1410
CTCCTAAATCAAGCATCTTTCTCTTACTGATGTTCAATAAAAAAATACGTTGCCAAGGCTCAAAAAA

In Fig. 3-14, the deduced amino acid sequence of the human enzyme is compared to the amino acid sequences of porcine uteroferrin and the bovine spleen tartrate-resistant acid phosphatase. Overall, there is 85% identity between the human enzyme and porcine uteroferrin. The similarity increases to 89% when only non-conservative substitutions are considered (Dayhoff, 1972). There is 82% identity between the human enzyme and the portions of the bovine enzyme which have been sequenced. A search of GenBank and the Protein Data Bank revealed no significant similarities between the cDNA sequence or inferred amino acid sequence of the human tartrate-resistant acid phosphatase and any other known DNA or protein sequences.

Northern Analysis

Northern analysis was carried out in order to determine the size of the mRNA coding for the human Type 5 phosphatase and to study the distribution of the enzyme in leukemic cells. A radiolabeled, single-stranded cDNA probe coding for the anti-sense strand of clone 6a was employed to analyze blots of RNA isolated from human placenta, normal human leukocytes, the leukocytes obtained from a patient with hairy cell leukemia, the human erythroleukemia cell line K562, the T-cell line JURKAT and the EBV-transformed B-cell line 1799ZR1.3 (Fig. 3-15). The probe detected a single transcript of approximately 1.5 kb in K562 cells (Fig. 3-15A and B) and 1799ZR1.3 cells (Fig 3-15C). The transcript detected in the hairy cells appeared to be slightly larger (Fig. 3-15A). This probe was also able to detect a 1.7 kb transcript in RNA isolated from the uterine endometrium of a day 60 pregnant pig (P.V. Malathy and R.M. Roberts, unpublished results). The transcript was not detected in

Fig.3-14 Comparison of the deduced amino acid sequence of the human tartrate-resistant acid phosphatase with the amino acid sequences of porcine uteroferrin and the bovine spleen acid phosphatase. Boxed regions indicate differences in the sequences. Blanks are unknown amino acid residues, X stands for either leucine or isoleucine in the beef spleen sequence; (-) indicates that adjacent residues are connected in the sequence; asterisks indicate potential glycosylation sites. The Asn at position 97 is known to be glycosylated in uteroferrin (Hunt et al., 1987). Most of the amino acid sequence for uteroferrin and all of the sequence for the beef spleen TR-AP is from (Hunt et al., 1987). Some regions in the porcine uteroferrin sequence (underlined) were deduced or interconnected from information obtained from uteroferrin cDNA clones (Simmen et al., 1988 and R. Simmen, unpublished results). A uteroferrin peptide sequence (KKILKR) placed tentatively after position 104 in Hunt et al. (1987) was not represented in the cDNA sequence. Its origin is unclear, although it is similar to the sequence SKISKR assigned to residues 105-110.

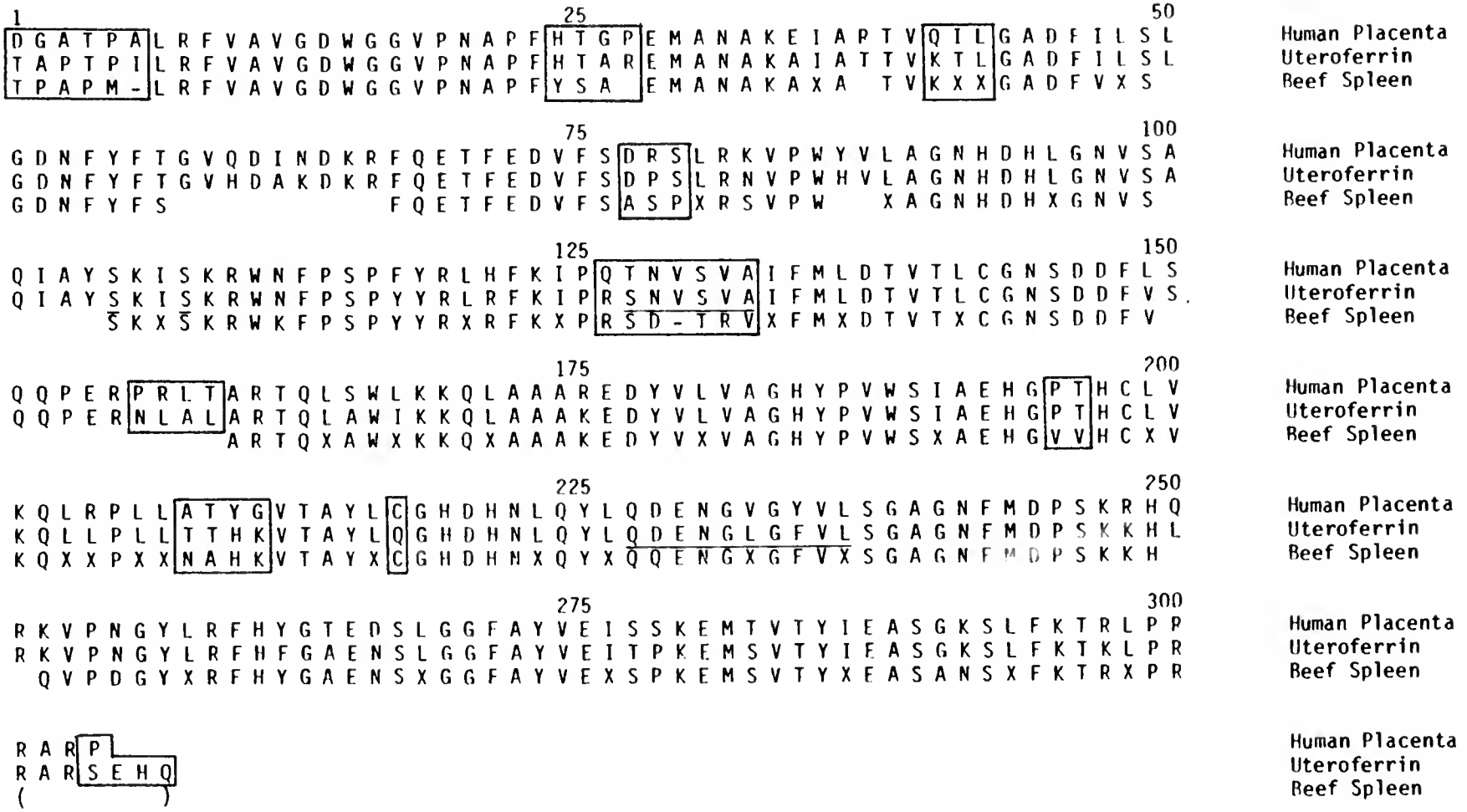
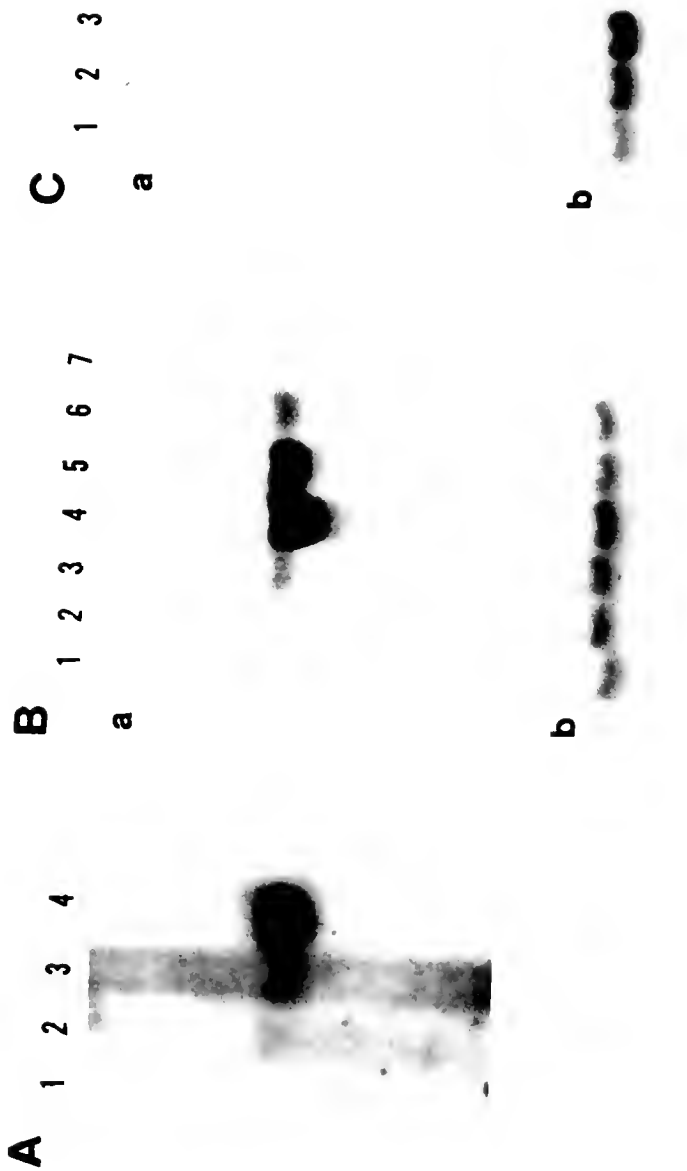

1
25
50
DGATPALRFVAVGDWGGVPNAPFHTGPEMANAKEIAPTVQILGADFILSL Human Placenta
TAPTPILRFVAVGDWGGVPNAPFHTAREMANAKAIATTVKTLGADFILSL Uteroferrin
TPAPM-LRFVAVGDWGGVPNAPFYSA EMANAKAXA TVKXXGADFVXS Beef Spleen
75
100
GDNFYFTGVQDINDKRFQETFEDVFSDRSLRKVPWYVLAGNHDHLGNVSA Human Placenta
GDNFYFTGVHDAKDKRFQETFEDVFSDPSLRNVPWHVLAGNHDHLGNVSA Uteroferrin
GDNFYFS FQETFEDVFSASPXRSVPW XAGNHDHXGNVS Beef Spleen
125
150
QIAYSKISKRWNFPSPFYRLHFKIPQTNVSVAIFMLDTVTLCGNSDDFLS Human Placenta
QIAYSKISKRWNFPSPYYRLRFKIPRSNVSVAIFMLDTVTLCGNSDDFVS Uteroferrin
SKXSKRWKFPSPYYRXRFKXPRSD-TRVXFMXDTVTXCGNSDDFV Beef Spleen
175
200
QQPERPRLTARTQLSWLKKQLAAAAREDYVLVAGHYPVWSIAEHGPTHCLV Human Placenta
QQPERNLALARTQLAWIKKQLAAAAKEDYVLVAGHYPVWSIAEHGPTHCLV Uteroferrin
ARTQXAWXKKQXAAAAKEDYVXVAGHYPVWSXAEHGVVHCXV Beef Spleen
225
250
KQLRPLLATYGVTAYLCGHDHNLQYLQDENGVGYVLSGAGNFMDPSKRHQ Human Placenta
KQLLPLLTTHKVTAYLQGHDHNLQYLQDENGLGFVLSGAGNFMDPSKKHL Uteroferrin
KQXXPXXNAHKVTAYXCGHDHNXQYXQQENGXGFVXSGAGNFMDPSKKH Beef Spleen
275
300
RKVPNGYLRFHYGTEDSLGGFAYVEISSKEMTVTYIEASGKSLFKTRLPR Human Placenta
RKVPNGYLRFHFGAENSLGGFAYVEITPKEMSVTYIEASGKSLFKTKLPR Uteroferrin
QVPDGYXRFHYGAENSXGGFAYVEXSPKEMSVTYXEASANSXFKTRXPR Beef Spleen
RARP Human Placenta
RARSEHQ Uteroferrin
( ) Beef Spleen

Fig. 3-15 Expression of tartrate-resistant acid phosphatase mRNA and its induction by TPA. Total RNA was isolated from the leukocytes of a normal individual and a patient with hairy cell leukemia; and from the cell lines K562, JURKAT and 1799ZR1.3. When indicated, the cell lines were cultured in the presence of 10^{-8}M 12-tetradecanoylphorbol 13-acetate as in Table 3-2. Northern blots of total RNA (40μg/lane) were hybridized to radiolabeled cDNA probes coding for either the human tartrate-resistant acid phosphatase (TR-AP) or β-actin (described in Materials and Methods). The size of the TR-AP mRNA is 1.5 kb and the β-actin mRNA is 2.0 kb as determined by comparison with RNA molecular weight standards. A, RNA was isolated from normal human leukocytes (lane 1), leukocytes of a patient with hairy cell leukemia (lane 2), K562 cells (lane 3) and K562 cells treated with TPA for 72 hours (lane 4). The Northern blot was probed with the cDNA coding for TR-AP. B, Lanes 1-7, respectively, contain RNA isolated from K562 cells treated with TPA for 0, 12, 24, 48, 72, 96 and 120 hours (see Table 3-2). In panel a, the Northern blot was probed with the cDNA coding for TR-AP; in panel b, the same blot was probed with the cDNA coding for β-actin. C, RNA was isolated from JURKAT cells (lane 1), JURKAT cells grown in the presence of TPA (lane 2) and the lymphoblastoid cell line 1799ZR1.3 (lane 3). In panel a, the Northern blot was probed with the cDNA coding for TR-AP; in panel b, the same blot was probed with the cDNA coding for β-actin.


A
1 2 3 4
B
1 2 3 4 5 6 7
a
b
C
1 2 3
a
b

normal human leukocytes (Fig. 3-15A) or placenta (data not shown) although leukocytes and placenta are known to contain the protein. Neither was the transcript apparent in the JURKAT cells (Fig. 3-15C), a T-cell acute lymphoblastoma leukemia cell line which does not express the Type 5 phosphatase (Drexler et al., 1987a).

Induction of the Tartrate-resistant Acid Phosphatase by TPA

When K562 cells were grown in the presence of 10^{-8}M TPA for 72 hours, a 30-fold increase in total acid phosphatase activity was observed (Table 3-2). The intracellular enzyme levels increased 25-fold by 72 hours, and declined thereafter. A change in morphology was also noted. With increased exposure to the phorbol ester, the cells increased in size and became irregular in shape.

Figure 3-15B shows that levels of mRNA coding for the human tartrate-resistant acid phosphatase increased dramatically after a 24 hour exposure to the phorbol ester and began to decline after 72 hours. Densitometry revealed that the mRNA was maximally induced 30-fold 72 hours after addition of the TPA. Clearly the increased expression of tartrate-resistant acid phosphatase activity in response to the phorbol ester is closely correlated with increased levels of mRNA coding for the enzyme. The signal for β-actin mRNA remained relatively unchanged as the time of exposure to the tumor promoter was extended.

The JURKAT cell line, which had undetectable levels of mRNA coding for the tartrate-resistant acid phosphatase (Fig. 3-15C), was also tested for tartrate-resistant acid phosphatase activity with and without the addition of TPA to the culture medium. Relatively low levels of tartrate-resistant acid phosphatase were detected in the cell lysates

TABLE 3-2

Tartrate-resistant acid phosphatase levels in K562 and JURKAT cells maintained on 10^{-8} TPA and K562 cells maintained on 60μM hemin

Cells	Treatment	Time (hours)	Units of Activity (mU/10^5 cells) Total Activity	Cells	Medium
K562	None	0	0.84	0.57	0.27
K562	10^{-8}M TPA	12	0.96	0.77	0.14
K562	10^{-8}M TPA	24	2.9	2.2	0.7
K562	10^{-8}M TPA	48	11.0	9.1	1.9
K562	10^{-8}M TPA	72	21.0	16.6	4.4
K562	10^{-8}M TPA	96	12.0	8.8	3.2
K562	10^{-8}M TPA	120	10.0	1.6	4.4
K562	60μM hemin	48	1.6	0.50	1.1
K562	60μM hemin	72	0.68	0.34	0.34
JURKAT	None	0	2.6	2.6	0
JURKAT	10^{-8}M TPA	72	2.6	1.1	1.5

Cells (10^6) were harvested at each time point and acid phosphatase activity measured in triplicate samples of medium and cell extracts from 10^5 cells. Each culture was maintained separately at a density of approximately 5 x 10^5 cells/ml by addition of fresh medium (see "Materials and Methods") containing 10^{-8}M phorbol ester or 60μM hemin when necessary. Cells were exposed to high salt/detergent buffer (Chapter 2), broken by dounce homogenization and the supension centrifuged at 14,000xg for 10 minutes. Activity in the cell lysate (supernatant fraction) and the culture medium was assayed with p-nitrophenylphosphate (20mM) as substrate in the presence of 2-mercaptoethanol (0.1M) and L-(+)-tartrate (0.1M). Results are expressed as milliunits (nmol substrate hydrolyzed 1 min. at 37°C) per 10^5 cells. The medium was not changed during the course of the experiment and so represents accumulated activity.

but not culture medium of JURKAT cells when TPA was not added to the culture (Table 3-2). However, 58% of the acid phosphatase activity appeared in the culture medium after 72 hours treatment with TPA, although the total amount of acid phosphatase activity did not change.

The Effects of Hemin on Acid Phosphatase Expression

When hemin is added to the culture medium of K562 cells, the cells express fetal and embryonic hemoglobins, and differentiate into erythrocyte-like cells (Rutherford et al., 1979). Since the tartrate-resistant acid phosphatase is expressed at relatively high levels in K562 cells, but is not found in erythrocytes, the effect of hemin on the expression of the Type 5 acid phosphatase in K562 cells was studied.

The effects of hemin were determined 48 and 72 hours after its addition to the medium. The cells changed drastically in morphology even 24 hours after hemin was added. K562 cells are normally spherical, regular in size and do not attach to the flask. In the presence of hemin the cells took on an irregular appearance, and there was some adherence to the flask. While the viability of log phase cultures of K562 cells, even in the presence of TPA, was always greater than 98%, the viability of K562 cells in the presence of hemin was consistently 90-95%. However, this loss of viability is unlikely to have influenced the results. Significantly, both the tartrate-resistant acid phosphatase activity (Table 3-2) and the mRNA level for the enzyme (data not shown) declined 72 hours after the addition of hemin.

Discussion

There may be several reasons why antibodies raised against porcine uteroferrin recognize human fibronectin. However, the possibility that

porcine fibronectin has contaminated uteroferrin preparations can be ruled out. Fibronectin is not a basic protein and its molecular weight is greater than 250,000, features which make it seem unlikely that fibronectin would co-purify with uteroferrin. Also, fibronectin has not been identified in the uterine secretions of pigs, at least in any appreciable quantity. Perhaps the most persuasive argument against fibronectin contamination, however, is the fact that two of the four monoclonal antibodies raised against tartrate-resistant acid phosphatases also recognized human fibronectin. Interestingly, when porcine uterine endometrium libraries were screened with anti-uteroferrin antibodies, fibronectin clones were never obtained. In contrast, all of the positive clones obtained by immunoscreening the human placenta cDNA library coded for fibronectin.

When the inferred amino acid sequence of fibronectin is compared to that of porcine uteroferrin, there are no obvious sequence similarities over long stretches of amino acids. There are, however, several regions of four to six amino acids which are common to both uteroferrin and fibronectin. It is possible that as few as four amino acids define a common epitope (see Atassi, 1976; 1983). These potential epitopes are shown in Table 3-3. It is interesting to note that of 13 potential epitopes, 9 of them are found in the 491 amino acid sequence coded by clone HP 6.1, and the remaining 4 epitopes are found in the 1834 amino acids not contained in HP 6.1.

The possibility that there is a structural feature common to both uteroferrin and fibronectin, not apparent by comparison of primary sequences, was examined. The domains encoded by amino acids 1709-2201

TABLE 3-3

Potential epitopes common to porcine uteroferrin and human fibronectin

Amino Acid Residue Numbers		Amino Acid Sequence	
Fibronectin	Uteroferrin	Fibronectin	Uteroferrin
1723-1729	274-280	VRVTPKE	VEITPKE
1736-1740	154-158	EINLA	ERNLA
1764-1769	136-140	LKDTLT	L DTVT
1780-1784	95-99	LENVS	LGNVS
1786-1790	298-303	PRRAR	PRRAR
1824-1828	4-8	TPIQR	TPILR
1878-1882	7-11	LRFLA	LRFVA
2064-2069	65-72	FQDTSE	FQETFE
2154-2157	11-14	AVGD	AVGD
39-42	95-98	LGNV	LGNV
32-36	151-155	QQPWR	QQPER
305-312	95-101	LGNGVSCQ	LGN VSAQ
1655-1660	213-218	TAELQG	TAYLQG
1693-1697	2-6	APTDL	APTPI

are made up of two types of repeats found in fibronectin. Clone HP 6.1 codes for 5 type III repeats and 2 type I repeats. This region of the protein is known to contain the heparin binding domain (Kornblihtt et al., 1985). The possibility that uteroferrin also contain a heparin binding domain was considered. Uteroferrin appeared to bind to heparin-agarose in a low salt buffer (0.01M NaCl), but could be eluted with a buffer containing 0.25M NaCl. True heparin binding proteins, such as the heparin-binding growth factors, elute only at very high salt concentrations, 1.0 to 1.6M (Lobb et al., 1986). Therefore, uteroferrin is not a true heparin binding protein, and it is not clear why anti-uteroferrin antibodies bind to human fibronectin.

When cDNA clones coding for porcine uteroferrin were employed to screen the same human placenta cDNA library, two cDNA clones coding for the human tartrate-resistant acid phosphatase were obtained. One of these clones, 6a, appears to be essentially full length. Examination of the 5'end of the sequence of 6a reveals two potential translation initiation sites (Fig. 3-13). The first site encountered, at nucleotides 91-97, is TGGATGG. This site, with the preferred G at position +4, is an initiation site of moderate strength (Kozak, 1986). The second site, at nucleotides 97-103, is GACATGT. This site has the preferred purine at position -3, and is also an initiation site of moderate strength (Kozak, 1986). Most likely both sites are employed; it cannot be assumed that initiation of translation occurs exclusively at the first ATG encountered (M. Kozak, personal communication).

The first amino acid of the mature protein is assigned as the aspartate residue coded by nucleotides 151-153, as predicted by Von

Heijne analysis (1983, 1985). The residue in position -1 (relative to aspartate +1) is the preferred alanine, and the serine at position -3 is the preferred small, neutral amino acid. Cleavage at this predicted point (marked by an arrow in Fig. 3-13) would infer a signal sequence of 17-19 amino acids, depending an which initiation codon was employed. Eukaryotic signal sequences typically range from 15-25 residues, with 19 amino acids the average length (Von Heijne, 1986). Cleavage before aspartate +1 would also produce a mature protein in closest agreement with the amino acid sequences of porcine uteroferrin and the bovine spleen enzyme (Fig. 3-14). Since the N-terminal sequence of the human tartrate-resistant acid phosphatase has not been determined, this assignment is tentative.

The mature human Type 5, tartrate-resistant acid phosphatase most likely consists of 304 amino acids, with an unglycosylated relative molecular mass of 34,193. This relative molecular mass is in good agreement with the apparent Mr of 34,000 for the mature protein, as determined by SDS-PAGE (Chapter 2). The apparent molecular weight of unglycosylated uteroferrin is 33,000, as measured by SDS-PAGE (Baumbach et al., 1984).

The human enzyme contains two potential attachment sites for N-linked oligosaccharides (boxed in Fig. 3-13). Both of these sites are conserved in porcine uteroferrin, but only one of them is found in the bovine spleen enzyme (Fig. 3-14). Each of these enzymes is known to be glycosylated (see Chapters 1 and 2).

The glycosylation site at Asn 97 of uteroferrin is known to be used in the Mr=35,000 form of the enzyme purified from allantoic fluid and

uterine flushes (Hunt et al., 1987). The second site, at Asn 128, may be employed in an Mr=37,000 form of uteroferrin which can be purified from uterine explant cultures and is found in the pink, high molecular weight uteroferrin heterodimer (Baumbach et al., 1986; Baumbach et al., manuscript in preparation). The extent of glycosylation of the human enzyme has not been determined, but the enzyme is known to be a glycoprotein (Chapter 2).

The tartrate-resistant acid phosphatases are known to contain two asymmetrical iron binding sites (see Chapters 1 and 2). It has been proposed that the iron atoms in these enzymes are coordinated to tyrosine and histidine residues (Gaber et al., 1979; Lauffer et al., 1983). Internal repeats containing histidine and tyrosine residues in identical spacial arrangements are not present in any of these phosphatases, which is consistent with non-identical environments for the iron atoms (Hunt et al., 1987). The residues that are ligands for the iron atoms cannot be assigned at this time, since the primary sequences of these iron-containing acid phosphatases contain several conserved histidine and tyrosine residues.

In order to study the distribution of the human tartrate-resistant acid phosphatase, Northern analysis was performed on three human leukocyte cell lines. A 1.5 kb transcript coding for this enzyme was detected in the human erythroleukemia line K562 and in the human B-cell line 1799ZR1.3. This transcript was not apparent in the human T-cell line JURKAT. These results are in good agreement with the work of Drexler et al. (1985, 1986, 1987a,b), who studied levels of tartrate-resistant acid phosphatase in a wide variety of leukemia cell lines by

means of isoelectric focusing. They determined that the tartrate-resistant acid phosphatase is generally expressed only in cells arrested late in differentiation. These researchers detected moderately high levels of tartrate-resistant acid phosphatase in K562 cells, but the enzyme was not detectable in JURKAT cells. Drexler et al. did not study the B-cell line 1799ZR1.3, but since it represents a B-cell in the later stages of differentiation (L. Smith, personal communication), it was expected that the cells would produce the enzyme. Of five EBV-transformed, late B-cell lines studied (the generous gift of Dr. Linda Smith), three produced high levels of the tartrate-resistant acid phosphatase (data not shown). These results are also in agreement with the work of Drexler et al. (1986).

The tumor-promoting phorbol ester TPA causes permanently established leukemia cell lines to differentiate to more mature stages (Kiss et al., 1987; Gazitt et al., 1987; Theil et al., 1987; Koeffler et al., 1981). The in vitro and in vivo effects of TPA are most probably mediated through the activation of protein kinase C and subsequent phosphorylation of cellular proteins (for a review, see Nishizuka, 1984). If expression of the tartrate-resistant acid phosphatase is indeed a differentiation-related event, its expression may be under the control of TPA and protein kinase C.

The effect of 10^{-8}M TPA on acid phosphatase levels in K562 cells was studied. K562 cells, unlike the closely related KG-1 cells, do not terminally differentiate into macrophage-like cells in the presence of TPA (Koeffler et al., 1981). Instead, they partially differentiate into megakaryoblastoid cells (Alitalo et al., 1988). Drexler et al. (1986)

have demonstrated qualitatively that K562 cells show a significant increase in tartrate-resistant acid phosphatase activity (as determined by isoelectric focusing) when treated with TPA for 72 hours. Thus, TPA may induce differentiation to a stage, or activation to a certain status, where acid phosphatase levels are elevated. It has been demonstrated in this chapter that TPA causes a marked increase in the quantity of tartrate-resistant acid phosphatase in K562 cells, both at the RNA and protein levels, although the effect is slow and possibly indirect. Interestingly, Gazitt and Polliack (1987) have demonstrated that a number of leukemia cell lines which were sensitive to the differentiating effect of TPA could be induced to differentiate terminally into cells reminiscent of hairy cells. They proposed that hairy cells (which are known to contain extremely high levels of the tartrate-resistant acid phosphatase) represent the terminally differentiated B-cell. Leukemia can be thought of as a disorder of differentiation. If the tartrate-resistant acid phosphatase is truly a marker of B-cell differentiation, the study of this enzyme's function and regulation may not only enhance our understanding of normal B-cell differentiation, it may also help to elucidate the relationships between the various leukemias.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

The experiments discussed in Chapters 2 and 3 clearly demonstrate that the human Type 5, tartrate-resistant acid phosphatase (TR-AP) belongs to the family of purple, iron-containing acid phosphatases, best characterized by porcine uteroferrin. In Chapter 2 a relatively simple protocol was described for the purification of the human enzyme. This protocol, based on the method established for the purification porcine uteroferrin (Chen et al., 1973), was also used to purify related enzymes from a variety of other sources. It would be interesting to determine whether the purple, manganese-containing acid phosphatases of plants could be purified by this method.

Much of the confusion about the properties of the human TR-AP (discussed in Chapter 1) has been resolved by the experiments in Chapters 2 and 3. The discrepancies about the activation of the enzyme with 2-mercaptoethanol and about the apparent molecular weight were adequately explained in Chapter 2. Apparently, uteroferrin and the other TR-APs exist as equilibrium mixtures of active (reduced) and inactive (oxidized) forms, and this equilibrium depends on the redox state of the fluid environment in which the enzymes are found. If this is the case, it is not surprising that the extent of activation of the TR-AP enzymes varied so much between preparations and throughout the purification process. It is interesting to note that while a stable, pink, high molecular weight, fully active form of uteroferrin exists,

there is no evidence of high molecular weight, fully active TR-AP enzymes from other sources such as rat, human or bovine spleen. The controversy over the enzymes' apparent "subunits" (found only in some cases) can be attributed to proteolytic degradation, since both uteroferrin and the beef spleen enzymes are single polypeptide chains (Hunt et al., 1987), and the human enzyme is coded for by a single mRNA species (Chapter 3). Unfortunately, the differences in substrate specificities between the Gaucher's spleen enzyme (Robinson and Glew, 1980; 1981) and the hairy cell spleen enzyme cannot be explained. The possibility that there are two or more genes coding for distinct TR-APs in the human cannot be ruled out, although chromosomal localization studies, carried out by means of in situ hybridization, revealed that the cDNA coding for the human placenta enzyme hybridized only to the long arm of chromosome 15 (B. Allen, C. Ketcham, H. Nick, unpublished results). The human bone (osteoclast) TR-AP has been purified by our laboratory (S. Allen et al., in press), and when N-terminal amino acid sequence is available, we will know whether the bone enzyme is identical to the placenta enzyme described in Chapter 3.

In Chapter 2, the production of monoclonal antibodies against both uteroferrin and the human enzyme was described. It was demonstrated that these antibodies cross-reacted with a wide variety of purple, iron-containing phosphatases. Furthermore, the four antibodies studied in detail did not compete with each other for binding to porcine uteroferrin, a result which indicated that each one recognized a different epitope. It is probable that four unique antibody binding sites do not represent the entire immunogenic repertoire of the native

uteroferrin molecule. Most likely, a mouse would not produce an antibody against a domain that its own TR-AP possesses, although one monoclonal antibody, 13.122, seemed to recognize a conserved epitope found on the TR-AP isozyme (see Chapter 2). From previous experience with a wide variety of proteins, Atassi (1976) proposed that a maximum of one immunogenic domain exists per 50 amino acids (about 5,000 daltons). Therefore, uteroferrin, a 307 amino acid protein with an unglycosylated apparent molecular weight of 33,000, could contain six or seven major immunogenic domains.

One set of competing monoclonal antibodies, represented by 5.122.10, 5.127.3 and 6.37.4, recognized an epitope that may be near the active site, since binding of the monoclonal antibody 5.127.3 inhibited uteroferrin's enzymatic activity. Perhaps this antibody could be useful in the identification of the peptide sequences around the active site. While it is believed that the iron atoms may be involved in substrate binding, and that a phosphoryl-enzyme intermediate may exist (see Chapter 1), the amino acid residues that make up the active site are unknown.

The monoclonal and polyclonal antibodies raised against uteroferrin were employed for the immunocytochemical localization of the human enzyme in placenta, bone tumors and white cells from patients with various types of leukemia. While our laboratory had been successful in localizing uteroferrin to the glandular epithelium in the porcine uterus (Fazleabas et al., 1984), we were unable to localize the human enzyme (C. Ketcham, M.K. Murray, T. Raub, R.M. Roberts, unpublished results). We consistently detected very little immunoreactive protein in the human

tissues and staining often appeared non-specific or diffuse. At least part of the problem may involve the fact that the polyclonal antibodies and some of the monoclonal antibodies cross-react with human fibronectin. The lack of success may also be attributed to the fact that the antibodies have a much lower affinity for the human enzyme than for uteroferrin.

Antibodies against the TR-APs may be useful for a number of clinically related projects. For example, the immunocytochemical identification of hairy cells with a polyclonal antibody raised against the human enzyme may be more specific and more sensitive than the histocytochemical methods now used. The antibodies may also be valuable diagnostically in the measurement of the levels of the TR-AP isozyme in plasma, by immunoassay. Echetebu et al. (1987) have demonstrated that anti-uteroferrin antibodies can be employed for the measurement of TR-AP levels in the sera of patients with Paget's disease and osteoporosis. These researchers were also able to detect the enzyme in human lung and Gaucher's spleen homogenates (but they could not detect the enzyme in placenta homogenate). Susan Allen, in collaboration with R.M. Roberts, has raised a monospecific polyclonal antibody against the human enzyme, and plans to develop a sensitive immunoassay for the detection of TR-AP in the sera of patients with osteoporosis.

The monoclonal antibodies were also tested as potential inhibitors of the phosphotransferase (discussed in Chapter 1) which transfers GlcNAc 1-phosphate to certain mannose residues of lysosomal enzymes. Unfortunately, none of the monoclonal antibodies, when bound to uteroferrin, were able to inhibit the phosphorylation of uteroferrin's

high mannose oligosaccharide chain (L. Lang, R.M. Roberts and S. Kornfeld, unpublished results). The region of uteroferrin (or any lysosomal enzyme) which is recognized by the phosphotransferase is still unknown. Identification of this region will be a major contribution to the field of lysosomal enzyme trafficking.

Despite the failure of the monoclonal antibodies to identify the region of uteroferrin recognized by the phosphotransferase, the TR-APs may still be useful for the study of the trafficking of lysosomal enzymes. The lysosomal bovine spleen enzyme is very similar in sequence to the secreted porcine uterine enzyme (Hunt et al., 1987; and Chapter 3). The most dissimilarity is noted around the N-terminal region and the second glycosylation site. It would be interesting to compare the sequences of cDNAs coding for the lysosomal versus secreted forms of the TR-AP enzyme in a single species (the cow or the pig, see Chapter 2). Perhaps the primary sequences of the secreted enzymes contain targeting information which directs them to secretory granules despite the fact that the phosphotransferase recognizes them as lysosomal enzymes, thus phosphorylating their high mannose oligosaccharides. The targeting of both secreted and lysosomal forms of TR-AP can be studied by transfection of their respective cDNAs into mammalian cell lines. Site directed mutagenesis can then be employed in an effort to redirect the enzymes, thus revealing the signals necessary for differential targeting.

An important topic which has not yet been addressed experimentally is the function of the human TR-AP. The persuasive evidence that uteroferrin functions in iron metabolism was presented in Chapter 1.

Although other functions have been proposed for other TR-APs, as a general phosphoprotein phosphatase (Davis et al., 1981) as a specific phosphotyrosyl phosphatase (Lau et al., 1985; 1987), or in nucleotide metabolism (Hara et al., 1984; 1985), there is little evidence to support these theories. Since it is believed that uteroferrin may function in iron metabolism, Shindelmeiser et al. (1987) considered the possibility that the bovine spleen enzyme might be involved in iron metabolism as well. Maybe the human TR-AP is also involved in iron metabolism. Some preliminary experiments could be done to determine whether or not this is the case.

The ideal cell line for these studies would be the human erythroleukemia K562 cells. Iron metabolism has been studied, to some extent, in these cells. They are known to synthesize both ferritin (Bottomley et al., 1985) and hemoglobin (Rutherford et al., 1979), and they express transferrin receptors (Van Rensounde et al., 1982). As shown in Chapter 3, these cells also express considerable amounts of TR-AP. Bottomley et al. (1985) have demonstrated that when K562 cells were incubated with [^{59}Fe] transferrin, up to 30% of the label could not be accounted for in hemoglobin, ferritin, or transferrin. Furthermore, the [^{59}Fe] from transferrin appeared in ferritin at a very slow rate, indicating that the iron taken up may have passed through one or more intermediates. Perhaps TR-AP is one of these intermediates.

It is widely believed that iron plays a critical role in cellular differentiation, cell growth and malignant transformation (see Gambari et al., 1986; Petraki et al., 1986). In K562 cells, differentiating agents caused a loss of transferrin receptors from the cell surface

(Petraki et al., 1986) and an increase in iron stores in ferritin (Mattia et al., 1986). The theory that TR-AP is a marker of lymphocyte differentiation was discussed in Chapter 3. It would be interesting to study the effects of perturbants of iron metabolism (such as chelators) and differentiating agents on TR-AP levels to determine whether there is any connection between TR-AP levels, iron metabolism and the differentiated state of the cells.

In conclusion, there is little doubt that uteroferrin and the other TR-APs will continue to be of interest to a wide variety of researchers. The unusual spectral properties of these proteins are currently being studied by physical biochemists interested in metalloprotein structure. The possible role of TR-APs in iron metabolism is presently being investigated by at least two groups of physiologists. Other clinically oriented laboratories are interested in the significance of TR-AP levels in osteoporosis and other bone disorders. Cell biologists will be interested in the differential targeting of the lysosomal versus secreted TR-APs. The regulation of uteroferrin's biosynthesis by steroid hormones is being studied by reproductive biologists and molecular biologists. It is therefore likely that the cDNA coding for the human TR-AP will be useful for future studies involving the distribution, function and regulation of the various TR-AP enzymes.

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BIOGRAPHICAL SKETCH

Catherine Mary Scopa was born on April 23, 1959, in Patchogue, New York. She is the fourth of the six children of Alfred and Antoinette Scopa. She grew up in nearby Port Jefferson, New York, where she attended a parochial elementary school and graduated from Earl L. Vandermuelen High School in 1977. Catherine attended Long Island University in Southampton, New York, and received a Bachelor of Arts degree in biology in 1981.

Later that year, she married Glenn Ketcham of Woodstock, New York. They moved to Gainesville, Florida, where Glenn pursued a graduate degree at the University of Florida, and Catherine obtained a technical position in the laboratory of Dr. R. Michael Roberts in the Department of Biochemistry and Molecular Biology.

In 1985, Catherine began her graduate studies under the supervision of Dr. Roberts. When Dr. Roberts left for the University of Missouri, Catherine completed her work in the laboratory of Dr. Harry S. Nick.

After Catherine completes the requirements for the Ph.D. degree, Catherine and Glenn will move to St. Louis, where Catherine will be a postdoctoral associate in the laboratory of Dr. Stuart Kornfeld at Washington University School of Medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R. Michael Roberts, Chairman
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry S. Nick, Cochairman
Assistant Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Fuller W. Bazer
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter M. McGuire
Assistant Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel L. Purich
Professor & Chairman of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael S. Kilberg
Associate Professor of Biochemistry
and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1988

Dean, College of Medicine

Dean, Graduate School